Hyperthermia-Induced Limitations and Adaptations to Exercise in the Heat

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Contents

Summary

Endurance exercise performance is impaired with acute exposure to warm environments but gradually recovers, at least partially, with heat acclimation (HA). Although investigated intensively for decades, both, the underlying physiological mechanisms by which hyperthermia impairs exercise performance and also by which these limitations can be overcome with HA remain elusive. Therefore, the overall purpose of the present PhD project was to investigate physiological mechanisms leading to diminished exercise capacity in the heat and how these may be altered with heat acclimatization.

Specifically, we hypothesised, that as a possible limitation, heat-induced hyperventilation may reduce arterial carbon dioxide partial pressure ($PaCO_2$) which in turn might lead to lower cerebral perfusion and oxygenation and thus to a decreased exercise performance. In order to test this hypothesis mechanistically we administered small amounts of CO_2 to the inspiration with the aim to prevent the hyperventilation-induced reductions in $PaCO_2$. Furthermore, we determined an extensive list of physiological variables to test their impact on the improved exercise capacity in the heat following HA. Special emphasis was placed on the role of a HA induced expansion of plasma volume. This was accomplished by conducting a meticulous counter-balanced cross-over heat training study. Additionally, since the determination of blood volume compartments, such as the plasma volume, is of uttermost importance for the outcome of these studies, the availability of a precise measuring method was indispensable. Therefore, a further goal of the present dissertation was to test the comparability of already established methods for detecting blood volume compartments and to determine the most accurate and feasible method to quantify alterations in blood volume compartments following an intervention.

Based on the conducted studies the key findings are summarized as follows:

1) While supplementation with CO_2 increased cerebral oxygenation this did not influence exercise performance in the heat. Hence, we concluded that hyperthermia-induced reduction in cerebral blood flow does not seem to be a limiting factor when exercising in the heat.

2) Plasma volume and exercise performance were both increased with HA. However, neither did those parameters correlate nor did artificial plasma volume expansion by albumin

beneficially influence exercise performance. Thus, an augmented plasma volume is likely not the main factor responsible for the increased exercise performance after HA.

3) In well-trained individuals, HA provoked physiological adaptations which facilitated exercise performance in hot but not in temperate conditions.

4) One blood volume compartment, such as plasma volume, can be indirectly estimated by the quantification of another compartment, and especially the carbon monoxide (CO) re-breathing proved to be an accurate and feasible method due to its low measurement error.

Zusammenfassung

In Kombination mit Hitzeexposition vermindert sich die Ausdauerleistungsfähigkeit. Diese kann sich aber durch Hitzeakklimatisation (HA) zumindest teilweise wieder verbessern. Trotz jahrzehntelanger Forschung bleibt jedoch bis heute unklar, welcher Mechanismus für die genannte Leistungsverminderung verantwortlich ist, und auch auf Grund welcher Adaptionen diese Limitierung durch HA teilweise umgangen werden kann. Daher war das Ziel der vorliegenden Dissertation, das Verständnis der Interaktion zwischen akuter und chronischer Hyperthermie und aerober Leistungsfähigkeit zu verbessern.

Spezifisch haben wir angenommen, dass, als ein möglicher limitierender Faktor, die Hitze-induzierte Hyperventilation den arteriellen Kohlenmonoxid Partialdruck ($PaCO_2$) senkt, sich damit die Hirndurchblutung und Oxygenierung verringert und dass sich dies wiederum negativ auf die Ausdauerleistungsfähigkeit auswirkt. Dies haben wir getestet, indem wir CO_2 zur Inspirationsluft zugeführt haben, um die Hyperventilation-induzierte Reduktion im $PaCO_2$ zu verhindern. Zusätzlich prüften wir mögliche physiologische Mechanismen, die nach HA zu einer verbesserten Ausdauerleistungsfähigkeit in der Hitze führen könnten und untersuchten ihre Auswirkungen auf die Ausdauerleistungsfähigkeit bei heissen und temperierten Bedingungen. Speziell haben wir uns dabei auf die Rolle eines erhöhten Plasma Volumens fokussiert. Zu diesem Zweck wurde eine ausführliche „counter-balanced, cross-over“ Hitze-Trainingsstudie durchgeführt. Da zusätzlich die Bestimmung von verschiedenen Blutvolumen-kompartimenten wie zum Beispiel dem Plasmavolumen für meine Studie von grosser Bedeutung war, war auch das Vorhandensein einer präzisen Messmethode unerlässlich. Daher bestand ein weiteres Ziel der vorliegenden Dissertation darin, verschiedene Messmethoden für die Bestimmung von Blutvolumenkompartimenten zu vergleichen und die präziseste und praktikabelste Methode zu finden, um Änderungen in verschiedenen Blutvolumen-Kompartimenten zu messen.

Zusammengefasst haben die in meinem Doktorat gesammelten Resultate folgende Haupterkenntnisse hervorgebracht:

1) Das Hinzugeben von CO_2 zur Inspirationsluft hat die Ausdauerleistungsfähigkeit nicht beeinflusst. Daraus schliessen wir, dass eine Hitze-induzierte Reduktion der Hirndurchblutung keinen limitierenden Einfluss auf die Ausdauerleistungsfähigkeit in der Hitze zu haben scheint.

2) Das Plasmavolumen ist nach HA erhöht. Trotzdem hat es weder mit der gleichzeitig erhöhten Ausdauerleistungsfähigkeit korreliert, noch hat sich eine künstliche Plasmavolumen Expansion förderlich auf die Ausdauerleistungsfähigkeit ausgewirkt. Daher scheint das erhöhte Plasma Volumen nicht für die erhöhte aerobe Leistungsfähigkeit nach HA verantwortlich zu sein.

3) In gut trainierten Personen hat HA physiologische Adaptionen ausgelöst, welche die Ausdauerleistungsfähigkeit in einer heissen Umgebung verbessert haben, jedoch nicht die Ausdauerleistungsfähigkeit bei temperierten Bedingungen.

4) Ein Blutvolumenkompartiment kann indirekt durch die Messung eines anderen Kompartiments bestimmt werden und besonders die Kohlenmonoxid-Rückatmungsmethode erwies sich wegen ihres tiefen Messfehlers als präzise und praktikabel.

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1. Introduction

1.1 Background

The combination of exercise or physical work and heat stress is longstanding and remains a relevant topic today as exemplified by the granting of several sports events such as the Olympic Games in Rio 2016 and the world cycling and soccer championships in Qatar 2016 and 2022, respectively, to countries with a hot and humid climate. Moreover, exposure to heat is also recognized as a severe constraint not only in athletes but also in the daily working life of for example military personnel on duty in the Middle East, firefighters or construction workers. Finally, as evidenced by the history of heat wave related deaths every summer, and this even in Europe (Bouchama, 2004), heat exposure is likewise a serious clinical problem for even non-exercising individuals, such as the elderly or ill people.

Also in research the physiological responses of the human organism to heat exposure have been extensively discussed for decades. Science is often driven by pragmatic needs, where solutions to real-world problems need to be found. This is certainly true with respect to human exposure to a hot environment, and one important industry which dominated early research was metal mining (Taylor, 2014). The work was driven by the mining industry and initiated by extremely high mortality rates in the early twentieth century (Cluver, 1932). Although these mines were not particularly hot (37.3 °C), the humidity approached 100 % due to water used for silica dust suppression which was the primary cause of heat related health problems (Cluver, 1932, Dreosti, 1950). Indeed, at least one death per month occurred from 1924 to 1932 (Cluver, 1932). Out of this necessity, heat acclimatization programs were developed to reduce the fatality rate due to heat stroke from >12 to <1 per year (Dreosti, 1950). From an exercise performance perspective, such heat acclimatization programs are not particularly important to reduce fatality rate but to preserve exercise capacity in the heat, as described in the subsequent chapters.

As it can be understood from the above, findings from hyperthermia and heat related research are also important in respect of social benefits and general health with and without exercise and can be directly implemented into praxis. The main purpose of the present PhD-project was to establish insights in regards to the interaction between exposure to heat and aerobic exercise. With the aim to determine the mechanisms facilitating hyperthermia-induced physiological limitations, we focused some of our work on “centrally mediated fatigue” – i.e. fatigue which predominantly is of cerebral origin and which recently has been proposed as a major obstacle to exercise in the heat. Additionally, to enhance the understanding of how to overcome these

limitations by heat acclimation (as induced by training in the heat) and to study the resulting implications for subsequent performance in normal ambient air temperature, old and new approaches were examined. Furthermore, commonly used methods for detecting heat training induced changes in blood volume compartments were compared.

After a brief review of the present state of knowledge in each respective field, the results of extensive experimental work are presented and discussed in four manuscripts, three of which to date are published (Keiser et al., 2013, Keiser et al., 2015a, Keiser et al., 2015b) in internationally peer-reviewed journals and whereof one is currently in submission.

1.2 Exercise in the heat

Individuals may exercise in a wide range of environmental conditions. While our cold tolerance is somewhat limited without significant support (clothing, equipment, and shelter), our capacity to withstand heat stress is well advanced (Taylor, 2014). Healthy humans normally regulate body core temperature near 37 °C at rest but it can increase until 41 °C during strenuous exercise without severe health related consequences (Sonna et al., 2000, Byrne et al., 2006). Increases in body core temperature above this range are associated with severe adverse health reactions or death (Bynum et al., 1978, Winkenwerder and Sawka, 2008). Hyperthermia can be defined as an increase in body core temperature above the set range specified for the normal active state of humans, which is ~37 °C at rest and ~38 °C during moderate exercise (IUPS, 1987). In such hyperthermic condition, the increased metabolic heat production associated with strenuous exercise, in combination with an impaired heat dissipation due to an elevated environmental temperature and/or humidity, generates a major physiological challenge for the exercising athlete. One of the most renowned cardiovascular physiologist Loring Rowell stated that "perhaps the greatest stress ever imposed on the human cardiovascular system (except from severe hemorrhage) is the combination of exercise and hyperthermia. Together these stresses can present life-threatening challenges especially in highly motivated athletes who drive themselves to extremes in hot environments" (Rowell, 1986).

A moderate increase in body core temperature and specifically in muscle temperature may beneficially influence exercise performance by increasing the speed of all chemical reactions. Accordingly, Asmussen and Bøje (Asmussen and Boje, 1945) showed a ~5 % increase in single sprint performance for every degree that muscle temperature was increased. In contrast, when

body core temperature is increased to a level where it induces hyperthermia, prolonged exercise performance is commonly decreased (Nybo, 2008).

The magnitude of heat stress and the resulting decrease in exercise performance depends on a complex interaction between environmental factors (e.g. temperature, humidity, wind and clothing), the individual's biologic characteristics (e.g. acclimatization status, fitness level, age, gender and size) and their activity level (metabolic rate, intensity and duration) (Sawka et al., 2012). Nonetheless, it has been shown that endurance performance is highest in relatively cool temperatures of around 10-14 °C and continuously decreases with increases in temperature (Figure 1) (Galloway and Maughan, 1997, Ely et al., 2007). Laboratory-based studies have demonstrated that the types of exercise which are impaired with hyperthermia are numerous, ranging from prolonged submaximal endurance performance (i.e. at intensities from 40 to 80 % $\dot{V}O_2$max and durations from ~1 h above) (Gonzalez-Alonso et al., 1999, Nybo et al., 2001, Lorenzo et al., 2010, Karlsen et al., 2015) up to maximal exercise lasting only ~3-10 min (Pirnay et al., 1970, Saltin et al., 1972, Gonzalez-Alonso and Calbet, 2003), repeated sprinting (Drust et al., 2005) and also isometric contraction (Nybo and Nielsen, 2001a, Morrison et al., 2004, Martin et al., 2005). Fewer studies are available which were conducted under field conditions but it has been suggested that for example also marathon running performance is reduced by approximately 1 min for each 1 °C increase in ambient air temperature beyond 8-15 °C (Zhang et al., 1992).

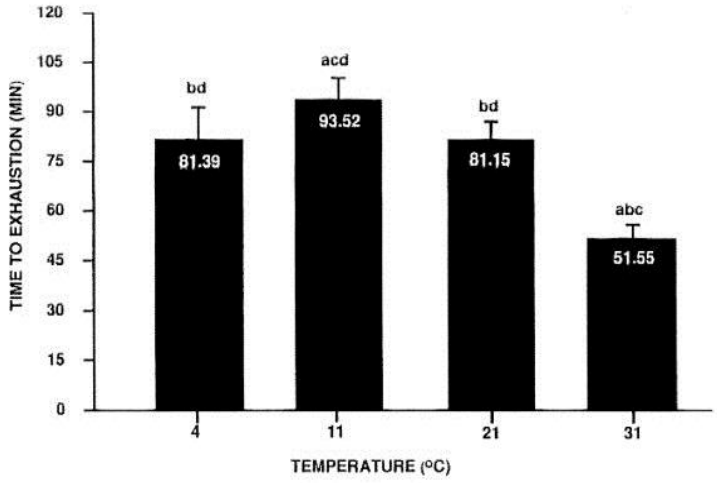

Figure 1. Time to exhaustion under the four different ambient air temperature conditions. Values are mean (SEM). A, b, c, d indicate a significant difference ($P < 0.05$) from corresponding values at 4 °C, 11 °C, 21 °C, and 31 °C, respectively. From Galloway and Maughan, 1997.

1.3 Physiological limitations to exercise in the heat

While, as described in the previous chapter, it is widely accepted that environmental heat stress impairs aerobic exercise performance, the actual mechanism or mechanisms by which this impairment occurs remains elusive (Cheung and Sleivert, 2004). The limitations to exercise in the heat have fascinated humans for decades. As early as 1916 Lee and Scott presented a detailed report about premature fatigue during exercise in the heat (Lee and Scott, 1916). Although experiments remained inconclusive and were conducted on cats this initial line of research provided a fascinating insight into the complexity of heat related studies during these times. As a conclusion Lee and Scott (1916) suggested the following mechanisms to be responsible for the hampered exercise performance in the heat: 1) circulatory changes – "drafting blood away from the brain and the muscles to the skin" (p. 487), 2) cerebral changes – "depression of cerebral activity" (p. 496), and 3) metabolic changes – "substances and products of abnormal metabolism act as fatigue substances" (p. 486). Astonishingly, these potential limitations to exercise in the heat are the same physiological mechanisms which researchers are still exploring today.

The most investigated system possibly leading to premature fatigue when exercising in the heat is likely the cardiovascular system. Especially the work of Rowell and colleagues significantly contributed to the understanding of what key role the cardiovascular system is playing in heat stressed exercising individuals (Rowell et al., 1966, Rowell, 1974, Rowell, 1983). Due to the demand for blood to both the exercising muscles and the skin for heat transfer, maintaining the required cardiac output ($\dot{Q}$) is challenged since blood volume is limited (Crandall and Gonzalez-Alonso, 2010). Together with dehydration and a reduced plasma volume (PV) resulting from sweating the cardiovascular system can rapidly reach its limits and premature fatigue occurs (Gonzalez-Alonso et al., 2008). However, since the mid-1990s researchers have also focused on the limiting influence of the central nervous system (CNS) on exercise performance in the heat (i.e. centrally mediated fatigue). The first convincing indication that the CNS may also excerpt an important role was provided by Nybo and Nielsen in 2001 (Nybo and Nielsen, 2001a). Compared to a control trial conducted in 18 °C, they found maximum voluntary contraction (MVC) force in active leg muscles to be reduced after a 50 min Time Trial at 40 °C. However, with superimposing electrical stimulation force production could be restored to levels observed in the control trial (Figure 2). Furthermore, MVC for the inactive arm muscles was reduced in the same way as for the leg muscle (Figure 3). Hence, they concluded that the ability to generate force during a prolonged MVC is impaired in hyperthermia, and that this is

associated to a reduction of centrally mediated feed forward mechanism. However, since this is a relatively new concept, the CNS and its role in hyperthermia induced fatigue has not been fully established yet. Therefore the first aim of the present dissertation focused on CNS derived limitations to exercise in the heat as described in the next paragraph.

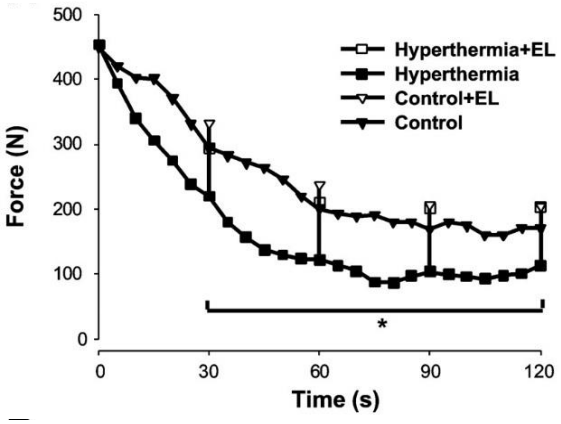

Figure 2. The changes in force during 2 min of sustained MVC with the knee extensors during hyperthermia and control. Maximal voluntary isometric contraction was attempted by the subjects throughout the 2 min, and electrical stimulation (EL) was superimposed every 30 s to assess the degree of central activation. Data are means ± SE for 8 subjects. *Significantly lower than control ($P < 0.05$). From Nybo and Nielsen, 2001a.

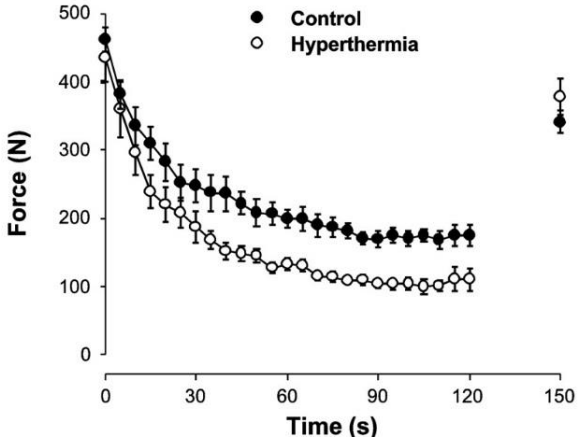

Figure 3. Changes in force during 2 min of sustained maximal handgrip contraction with or without exercise-induced hyperthermia. Data are means ± SE for 8 subjects. From Nybo and Nielsen, 2001a.

Aim 1: CNS derived limitations to exercise in the heat

While the experiments conducted by Nybo and Nielsen (Nybo and Nielsen, 2001a) have shown that perturbations in the CNS should also be considered as a limiting factor when exercising in the heat, the underlying mechanism(s) still remain unknown. Nybo and Nielsen suggested one possible explanation to be a reduced cerebral blood flow (CBF) (Nybo and Nielsen, 2001a), as indicated by a reduced middle cerebral artery velocity ($MCAv_{mean}$). The initial idea was that hyperthermia induced hyperventilation could reduce arterial carbon dioxide partial pressure ($PaCO_2$) to such an extent were it favours cerebral vasoconstriction resulting in a reduced brain perfusion. Indeed, in 2001 Nybo and Nielsen confirmed this association as they showed similar declines in $PaCO_2$ and $MCAv_{mean}$ in heat-stressed humans during exercise (Nybo and Nielsen, 2001b) (Figure 4).

A reduction of $MCAv_{mean}$ during exercise in the heat has been proposed to limit exercise performance by two main mechanisms (Nybo, 2010): i) In goats, it has been demonstrated that selective brain cooling significantly increased exercise capacity (Caputa et al., 1986). Accordingly, it can be speculated that a reduced $MCAv_{mean}$ might limit the cooling effect of inflowing blood and thereby lead to a rise in brain temperature and subsequent fatigue (Nybo et al., 2002). ii) As a consequence of the reduced cerebral perfusion, O_2 delivery to the cerebellum might become insufficient which in turn could lead to cerebral deoxygenation and centrally mediated fatigue (Amann and Kayser, 2009, Rasmussen et al., 2010). Although augmenting $MCAv_{mean}$ in hypoxic (Subudhi et al., 2011, Fan et al., 2012, Siebenmann et al., 2013) and normoxic (Subudhi et al., 2011, Flück et al., 2014) conditions did not affect exercise performance, this remains unknown for hyperthermic conditions.

Therefore, the first aim of my PhD project was to elaborate on the hypothesis that a decreased CBF, secondary to a reduction in $PaCO_2$, leads to an impaired exercise performance in the heat. This was done by adding small amounts of CO_2 to the inspiration of study volunteers to prevent the drop in $PaCO_2$, and therefore the concomitant drop in $MCAv_{mean}$, during an incremental exercise test. The collected results are enclosed in the first manuscript which is presently published in a peer-reviewed journal (Keiser et al., 2015b).

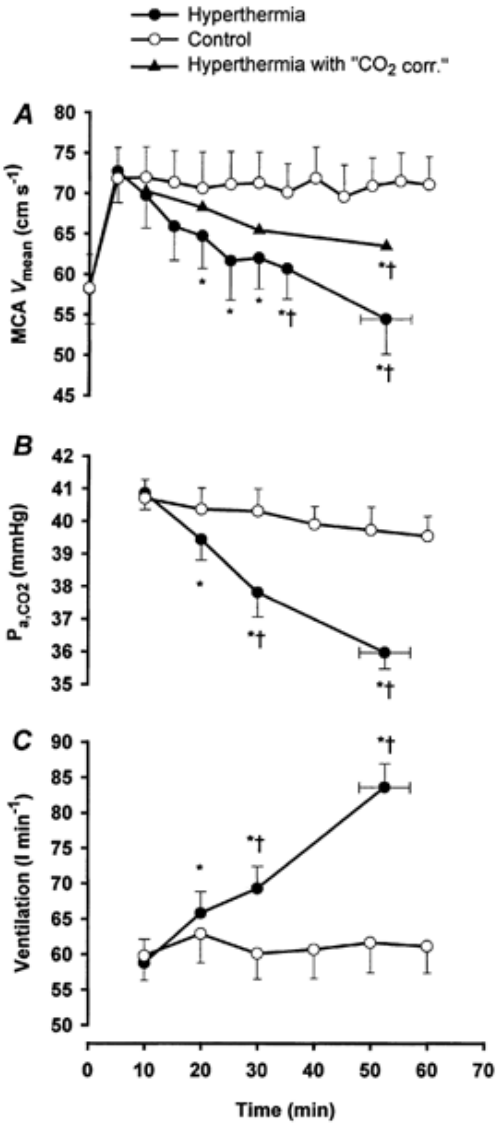

Figure 4. *A*, MCA V_{mean} (middle cerebral artery mean blood velocity) during prolonged exercise in control (○), during hyperthermia (●) and when the values were 'corrected' and related to the $P_{a,CO2}$ in the control trial (▲). *B*, $P_{a,CO2}$ calculated arterial caron dioxide pressure in control (○) and during hyperthermia (●). *C*, ventilation in control (○) and during hyperthermia (●). Values are means ± S.E.M. for 8 subjects. *Significantly different from 10 min value, $P < 0.05$; †significantly different from control, $P < 0.05$. From Nybo and Nielsen, 2001b.

1.4 Exercise performance and heat acclimation

Beside pre-cooling and fluid intake, continuous exposures to a hot environment are likely the most common and efficient way to, at least partially, overcome the hyperthermia induced limitations (Quod et al., 2006). Continuous exposures to a hot climate (i.e. acclimatization) or artificially hot environment (i.e. acclimation), hereafter collectively named HA, has repeatedly proven to increase exercise performance in the heat and reduce heat stress on the human organism (Bass et al., 1958, Nadel et al., 1974, Nielsen et al., 1997, Lorenzo et al., 2010, Karlsen et al., 2015).

As mentioned earlier, the roots of systematic heat acclimation programs date back to the early 20th century where many mine laborers suffered from severe health related arduousness with the hot and especially humid environment that they worked in (Cluver, 1932). Droesti was first to develop a substantial heat acclimatization program for novice mine laborers which tremendously reduced fatality rate (Dreosti, 1935, Dreosti, 1950). Moreover, the strategy of combining physical work or exercise with heat exposure was initiated (Eichna et al., 1945, Wyndham et al., 1953). Encouraged by the success of these acclimation programs, Wyndham and Strydom (Wyndham and Strydom, 1969) eventually developed a well elaborated acclimatization program including above-ground climatic rooms in which many miners could be simultaneously heat adapted and where underground work was replaced by bench stepping. Additionally, with advanced acclimation, exercise performance (i.e. stepping rate) was continuously increased to reach a progressive physiological overload (Wyndham and Strydom, 1969). With the implementation of such procedures Wyndham and Strydom cleared the way for specialised HA programs. Furthermore, already in these times, they recognised the importance of including physical activity and the need of progressively disturbing homeostatic balance by continuously increasing physical stress with progressive acclimation to elicit the best possible physiological adaptations. These two points are today still considered as two main points when designing an optimal HA program (Adolph, 1956, Taylor, 2014).

Although HA and its physiological adjustments have been investigated for centuries (Pandolf et al., 1977, Shvartz et al., 1977, Cadarette et al., 1984) many important points still remain unclear. Therefore, aim 2 and 3 of my PhD project were both related to physiological adaptations to HA and their effect on exercise performance, as specified in the following paragraphs.

Aim 2: Physiological adaptations leading to an enhanced exercise performance after repeated training in the heat

It is generally recognised that HA elicits physiological adaptations which enhance exercise performance in the heat (Sawka et al., 1985, Nielsen et al., 1993, Lorenzo et al., 2010) even in well-trained individuals (Lorenzo et al., 2010, Karlsen et al., 2015, Keiser et al., 2015a). Although the underlying physiological adaptations responsible for the increased exercise performance have been investigated for centuries (Pandolf et al., 1977, Shvartz et al., 1977, Cadarette et al., 1984) they remain somewhat elusive. The most prominent candidate is likely the hyperthermia induced expansion of PV (Senay, 1975, Nielsen et al., 1993, Lorenzo et al., 2010). An increased PV could explain several improvements which are usually described to occur with HA: an increased sweat output (Senay et al., 1976, Nielsen et al., 1997), an increased maximal cardiac output (Lorenzo et al., 2010), an increased stroke volume and therefore a reduced submaximal heart rate (Wyndham et al., 1968, Senay et al., 1976, Nielsen et al., 1997) and a reduced submaximal core temperature (Senay et al., 1976) which all could potentially enhance exercise performance. Additionally, due to an increase in PV, HA might also diminish hyperthermia-induced reduction of cerebral blood flow that may accelerate centrally mediated fatigue (Nybo, 2008, Rasmussen et al., 2010) and also the cooling of the cerebellum could be facilitated with an enhanced brain perfusion as suggested by Nybo et al. (Nybo et al., 2002). Although this argumentation sounds logic, acute expansion of PV has failed to reduce heart rate and core temperature during submaximal walking (Sawka et al., 1983) and cycling (Watt et al., 2000) in the heat, arguing against an elevated PV to be the main responsible factor for the enhanced exercise performance after HA. Since these experiments only included submaximal exercise performance, studies covering also maximal exercise performance, however, are lacking.

Further adaptive responses to repeated heat exposure which may favour exercise performance in the heat are proposed to include a more pronounced sweating response and a reduced loss of electrolytes in the sweat (Kirby and Convertino, 1986, Nielsen et al., 1997). While this appears to be of minor importance for near maximal or maximal intensity exercise challenges it might be favorable for prolonged submaximal exercise performance.

Thus, the second aim was to elaborate on the physiological mechanisms leading to improved exercise capacity in the heat following HA. Special emphasis was placed on modifications in PV and their influence on exercise performance in the heat. For these purposes, physiological

adaptations including changes in PV, $\dot{Q}$, heart rate, rectal temperature, $MCAv_{mean}$, sweat rate and sweat electrolytes after 10 days of heat training were assessed and PV was acutely expanded by means of albumin infusion to simulate the increased PV after a HA period.

Aim 3: Influence of repeated training in the heat on exercise performance in temperate conditions

As described above, it is widely accepted that with HA exercise performance in the heat gradually recovers (Sawka et al., 1985, Nielsen et al., 1993, Lorenzo et al., 2010). In addition to this, a few years ago the idea was raised that HA induced alterations in $\dot{Q}$, stroke volume, heart rate, core temperature, and/or fluid balance might not only have ergogenic effects on exercise performance in hot but also in temperate or cool conditions (Lorenzo et al., 2010, Corbett et al., 2014, Periard and Racinais, 2015). In a recent review Corbett et al. (Corbett et al., 2014) analysed several studies investigating the effect of HA on exercise performance in a temperate and cool environment. Although some studies have reported an enhanced exercise performance after HA in temperate (21-21.5 °C) (Nadel et al., 1974, Sawka et al., 1985) and cool (13 °C) (Lorenzo et al., 2010) conditions, most of the findings have been influenced by numerous confounding factors such as the absence of a control group, the inclusion of untrained subjects, suboptimal HA programs, or the application of an unclear study design. Probably the most convincing study mentioned in this review was conducted by Lorenzo et al. (Lorenzo et al., 2010). After 10 days of HA in well trained subjects ($\dot{V}O_2max$ ~67 $ml.min^{-1}.kg^{-1}$) they found $\dot{V}O_2max$ and Time Trial performance to be improved by 5 and 6 %, respectively, when tested in 13 °C ambient temperature (Lorenzo et al., 2010). Yet, it needs to be mentioned that the training sessions in the heat were conducted at a relatively higher exercise intensity as compared to a control training and therefore, it cannot be ruled out that at least some of the observed effects were due to the more intense training. Accordingly, it has been shown that heat training conducted in endurance athletes and appropriate control subjects did not further enhance normothermic $\dot{V}O_2max$ or outdoor Time Trial performance (Karlsen et al., 2015). Therefore the third aim was to test whether in well-trained subjects heat training facilitates exercise performance not only in hot but also in temperate conditions. This was done by assessing exercise performance at 38 and 18 °C before and after 10 days of HA.

The study aims 2 and 3 were together addressed in a meticulous counter-balanced cross-over HA study where exercise performance and various physiological adaptations were assessed

before and after 10 days of heat training in 18 and 38 °C. The obtained outcomes resulted in one combined manuscript which is to date published in a peer-reviewed journal (Keiser et al., 2015a).

1.5 Determination of blood volume compartments

Detecting changes in different blood volume compartments is important for clinical as well as for scientific purposes. With altitude training for example researchers might be especially interested in its potential effect on hemoglobin mass (Hb_{mass}) or red blood cell volume (RBCV) (Wehrlin et al., 2006, Robach et al., 2012, Siebenmann et al., 2012). During my PhD studies, on the other hand, I was particularly interested in heat training induced alterations of PV and hence the availability of a reliable and precise method to determine blood volume compartments is essential. This can not only be achieved by comparing different measuring methods but also by improving already existing procedures.

To date several methods are used for measuring different blood volume compartments and all of these are based on the same dilution principle: the volume of a compartment can be calculated from the concentration of an administrated tracer that is restricted to that specific compartment. The remaining compartments can then easily be quantified by the inclusion of the hematocrit (hct) and/or the hemoglobin concentration ([Hb]). However, this integration comprises certain errors since it has been shown that venous hct does not equal systemic hct. The reason for this discrepancy is likely related to a noncirculating part of PV which is not included in the venous hct measurements. Recent intravital microscoping images revealed that there are three compartments of total blood volume (BV): 1) RBCV, 2) a circulating part of PV, and 3) a noncirculating part of PV, which is bound by the endothelial glycocalyx at the endothelial surface layer (ESL) (Vink and Duling, 1996). This exclusion zone for flowing red blood cells is in dynamic equilibrium with the flowing plasma (Pries et al., 2000) and shows a volume of around 350 ml (Jacob et al., 2007). Therefore, a blood sample obtained from an arm vein overestimates the real systemic hct. To at least partially overcome this limitation a systemic hct/venous hct factor of 0.91 has been implemented (Burge and Skinner, 1995). In reality, however, the ratio may vary from 0.73 to 1.10 and therefore blood volumes which are estimated from the determination of another blood compartment may not necessarily be true.

Theoretically, if different blood volume compartments are to be measured, different methods should be applied for separately determining each blood volume compartment with its specific tracer. However, if only one method could be used for measuring all different blood volume compartments this would considerably safe time and money. Therefore, as explained below, the fourth aim addressed in my dissertation was to compare different commonly applied methods to measure blood volume compartments, namely the carbon monoxide (CO) re-breathing, the Indocyanine Green (ICG) and the Sodium Fluorescein (SoF) method, to see if one blood volume compartment could be indirectly estimated by the quantification of another compartment and to find the most accurate and feasible method for detecting alterations in different blood volume compartments.

Aim 4: Optimisation and comparison of methods used to determine blood volume compartments

For heat related studies especially changes in PV are of importance. Direct determination of PV was for a long time done by using the dye Evans Blue which binds to the protein albumin. The use was eventually abandoned due to unacceptable levels of patient discoloration and reports of sensitivity reactions and carcinogenicity in animals. Albumin can also be tagged with a radioactive tracer. A radioactive tracer approach offers many advantages, but unfortunately the inherent dangers of radioactive isotopes may make the methodology unsuitable. There are alternatives to Evans Blue and radioactive labelling; indocyanine green (ICG) dye and the fluorescent Texas Red (so far only used in rats) (Gillen et al., 1991) where ICG today seems the most relevant. ICG is a water soluble tracer which rapidly binds to plasma proteins (mainly albumin) and its light absorption can easily be determined by standard spectroscopy. It is frequently used for measuring $\dot{Q}$ and liver blood flow but in 1968 was also first successfully applied for determining intravascular volumes (Bradley and Barr, 1968). Using fluorescence videomicroscopy of skin capillaries it has been shown that ICG enters the ESL within 1 min after infusion (Bollinger et al., 1991, Brulisauer and Bollinger, 1991), and hence allows for the determination for both circulating and non-circulating plasma.

For the quantification of Hb_{mass}, the most frequently used method is the CO re-breathing where CO bound to hemoglobin is used as a marker. Although measurements with CO have already been done in the 19th century (Gréhant and Quinquaud, 1882), it was not before 1990 when Fogh-Andersen and Thomsen developed a routinely applicable method for detecting Hb_{mass}

with the use of CO (Fogh-Andersen et al., 1990, Thomsen et al., 1991). Later, this method has been further improved by Burge and Skinner (Burge and Skinner, 1995). Today two different methods of the CO re-breathing exist, the original 10 min version (Burge and Skinner, 1995) and a short version introduced by Schmidt and Prommer (Schmidt and Prommer, 2005). The problem with all dilution based methods is the need for a uniform distribution of the tracer throughout the body. This might be jeopardised since the CO re-breathing procedures are usually conducted in a seated position. In this seated position blood is pooled in the large leg veins which might not be tagged with CO during the re-breathing period (Wennesland et al., 1962) and in turn leading to an underestimation of Hb_{mass}. This shortcoming might be overcome if venous return is facilitated during the re-breathing procedure e.g. by the inclusion of light exercise or passive tilting. If successful, this might not only lead to a better validity but also an even better reproducibility of the CO re-breathing method which could make it an appealing alternative for not only sensing alterations in Hb_{mass} but also for detecting HA induced changes in PV.

A further method that might proof as a good method for measuring blood volume compartments is the Sodium Fluorescein (SoF) method where red blood cells are stained with SoF. This has been considered as a good alternative to radioactive tracers such as ^{51}Cr or ^{99}mTc. First experiments with fluorescent cells were conducted by Hansen where blood volume of animals was successfully determined by fluorescein isothicyanate (FITC) labeled erythrocytes (Hansen, 1985). However, FITC cannot be used for humans. A few years later, Lauermann in 1991 was first to use SoF (Lauermann, 1991), a dye mostly used in ophthalmology (Yannuzzi et al., 1986) and Haller et al. further improved this method by replacing the manual red cell counting by flow cytometry (Haller et al., 1997). So far this method is mainly used in clinical settings (Orth et al., 1998) and the feasibility for research purposes remains to be established.

Therefore, the fourth study aim of my PhD project was i) to further increase the validity and precision of the already well established CO re-breathing technique and ii) to determine whether one blood compartment can be indirectly estimated by quantification of another compartment and to test which method could be considered as the most feasible and accurate method for detecting HA induced changes in blood volume compartments. This was done by comparing different methods commonly used for the quantification of blood volumes and Hb_{mass}. The observations are presented in two manuscripts one of which is to date published in a peer-reviewed journal (Keiser et al., 2013) whereas the other is currently in submission.

2. Manuscripts

2.1 Restoring heat stress-associated reduction in middle cerebral artery velocity does not reduce fatigue in the heat

2.2 Heat training increases exercise capacity in hot but not in temperate conditions: a mechanistic counter-balanced cross-over study

2.3 The carbon monoxide re-breathing method can underestimate Hb_{mass} due to incomplete blood mixing

2.4 Detection of blood volumes and Hb_{mass} by means of CO re-breathing and Indocyanine Green (ICG) and Sodium Fluorescein (SoF) injections

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Restoring heat stress-associated reduction in middle cerebral artery velocity does not reduce fatigue in the heat

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Heat-induced hyperventilation may reduce $PaCO_2$ and thereby cerebral perfusion and oxygenation and in turn exercise performance. To test this hypothesis, eight volunteers completed three incremental exercise tests to exhaustion: (a) 18 °C ambient temperature (CON); (b) 38 °C (HEAT); and (c) 38 °C with addition of CO_2 to inspiration to prevent the hyperventilation-induced reduction in $PaCO_2$ (HEAT + CO_2). In HEAT and HEAT + CO_2, rectal temperature was elevated prior to the exercise tests by means of hot water submersion and was higher ($P < 0.05$) than in CON. Compared with CON, ventilation was elevated ($P < 0.01$), and hence, $PaCO_2$ reduced in HEAT. This caused a reduction ($P < 0.05$) in mean cerebral artery velocity ($MCAv_{mean}$) from 68.6 ± 15.5 to 53.9 ± 10.0 cm/s, which was completely restored in HEAT + CO_2 (68.8 ± 5.8 cm/s). Cerebral oxygenation followed a similar pattern. $\dot{V}O_2$max was 4.6 ± 0.1 L/min in CON and decreased ($P < 0.05$) to 4.1 ± 0.2 L/min in HEAT and remained reduced in HEAT + CO_2 (4.1 ± 0.2 L/min). Despite normalization of $MCAv_{mean}$ and cerebral oxygenation in HEAT + CO_2, this did not improve exercise performance, and thus, the reduced $MCAv_{mean}$ in HEAT does not seem to limit exercise performance.

Humans regulate body core temperature (T_{core}) near 37 °C and fluctuations within 35–41 °C are generally well tolerated by most humans. However, increasing T_{core} may have multiple physiological consequences including a diminished endurance exercise capacity. Such a diminishment in endurance capacity has been demonstrated in multiple environmental settings which enhance T_{core}. Marathon running performance, for example is reduced by approximately 1 min for each 1 °C increase in ambient air temperature beyond 8–15 °C (Zhang et al., 1992) and in laboratory conditions, exercise time to exhaustion is reported 45% longer at 11 °C when compared with 31 °C (Galloway & Maughan, 1997).

The underlying physiological mechanisms by which an elevated T_{core} limits exercise performance remains elusive although investigated intensively for decades (Gonzalez-Alonso et al., 1999; Nybo et al., 2014). Classically the reduction in performance has been attributed to a redistribution of blood flow away from the active skeletal muscles and toward the skin in favor of thermoregulation (Rowell, 1974; Crandall & Gonzalez-Alonso, 2010). The first convincing evidence that the central nervous system (CNS) may also excerpt an important role, however, was provided by Nybo and Nielsen (2001a) in that they demonstrated a reduction in maximum voluntary contraction (MVC) force in active leg and inactive arm muscles during exercise in the heat. The results were later confirmed (Drust et al., 2005), and it has subsequently been demonstrated that the impairments in MVC are related to elevations of T_{core} rather than to skin or local muscle temperature (Thomas et al., 2006). However, whether these measures of centrally mediated fatigue performed during static exercise are of relevance to dynamic exercise limitations remains unknown. Notwithstanding, the involvement of the CNS in heat stress-induced fatigue development has been hypothesized as being linked to the diminished cerebral perfusion and oxygenation during such exercise. Rowell and Blackmon (1986) initially suggested that cerebral perfusion and oxygenation could become limiting factors when the heat stress-induced hyperventilation, and hence drop in arterial carbon dioxide partial pressure ($PaCO_2$), would reach magnitudes favoring cerebral vasoconstriction. Nybo and Nielsen (2001b) confirmed the association as they observed parallel declines in $PaCO_2$ and mean middle cerebral artery velocity [$MCAv_{mean}$; a surrogate measure for cerebral blood flow (CBF)] in heat-stressed humans during exercise. One aim with the present study was to elaborate on this observation by preventing the decrease in $PaCO_2$ through the addition of CO_2 to the inspiration of exercising and heat-stressed humans, and thereby test the hypothesis that this

would abolish the reduction in $MCAv_{mean}$. The importance for heat-induced changes in $PaCO_2$ to affect $MCAv_{mean}$ has been demonstrated during passive heat stress (Brothers et al., 2009; Nelson et al., 2011; Bain et al., 2013) and during submaximal exercise (Rasmussen et al., 2004), but without a control heat trial and therefore the influence of a decrease in CBF on performance in the heat still remains unknown.

A reduction of $MCAv_{mean}$ during exercise in the heat has been proposed to induce exercise limitations by several mechanisms (see also Nybo, 2010 for complete review): (a) In the goat, selective brain cooling, by means of changing the temperature of implanted cooling elements, was demonstrated to augment exercise capacity (Caputa et al., 1986). Accordingly, it has been speculated that a reduced $MCAv_{mean}$ would limit the cooling effect of inflowing blood and thereby lead to a rise in brain temperature and subsequent fatigue. (b) Centrally mediated fatigue may be promoted by an insufficient O_2 delivery to the brain (Amann & Kayser, 2009; Rasmussen et al., 2010), which could become the consequence when cerebral perfusion is reduced. However, recent studies augmenting $MCAv_{mean}$ in hypoxic (Subudhi et al., 2011; Fan et al., 2012; Siebenmann et al., 2013) and normoxic (Subudhi et al., 2011; Flück et al., 2014) conditions have demonstrated that exercise capacity is not affected by changes in cerebral oxygenation, suggesting that this might also not be the case in heat-stressed humans. Nonetheless, this remains unknown.

The aims of the study thus were to (a) establish whether $MCAv_{mean}$ is reduced during exercise in the heat secondary to a reduction in $PaCO_2$, and (b) test the hypothesis that normalization of $MCAv_{mean}$ does not lead to concomitant improvements in exercise performance.

Methods

Participants

Eight moderately trained men (24 ± 2 years, 74 ± 3 kg, 182 ± 6 cm, $\dot{V}O_2max$ 61.2 ± 4.4 mL/kg/min, ± standard deviation) were recruited to participate in the study. All experimental protocols and procedures were approved by the ethical committee of the Swiss Federal Institute of Technology Zurich (EK 2013-N-23) and conformed to the Declaration of Helsinki. Prior to participation oral and written informed consent was obtained from each participant. All participants refrained from exercise for 24 h, alcohol and caffeine for 12 h prior to the study, and fulfilled the inclusion criterion of a $\dot{V}O_2max > 55\ mL/kg/min$.

Study design

All participants completed a preliminary maximal exercise test in a cool environment (18 °C) to become familiar with the experimental set-up. The exercise test was performed on a cycle ergometer (Monark Ergomedic 839 E, Monark Exercise AB, Varberg, Sweden) equipped with a triathlon handlebar. Thereafter, participants visited the laboratory on three occasions to conduct $\dot{V}O_2max$ tests once when acutely exposed to 18 °C (30% humidity, CON) and twice at 38 °C (30% humidity) breathing either ambient air (HEAT) or CO_2-enriched air (HEAT + CO_2). In the latter, the inspired CO_2 fraction was continuously adjusted from the beginning (Altitrainer, SMTEC, Nyon, Switzerland) to maintain $PaCO_2 > 40$ mmHg. The amount of CO_2 added to the inspiration was manually adjusted based on breath-by-breath feedback of $PetCO_2$. $PaCO_2$ was estimated from end-tidal PCO_2 ($PetCO_2$) by the equation of Jones (Jones et al., 1979).

The order of the trials was randomized and blinded. All test sessions followed the same protocol and were separated by at least 48 h.

Protocol

Prior to each $\dot{V}O_2max$ test participants were immersed into a whole-body water bath for 19 ± 1 min. While prior to the CON testing, the water temperature was thermoneutral (~34 °C), it was ~42 °C prior to the HEAT and HEAT + CO_2 trials. In the HEAT and HEAT + CO_2 trials this allowed increasing ($P < 0.05$) participants' rectal (T_{rec}) by 0.6 °C without employing exercise, whereas in the CON trial T_{rec} remained unaffected. An elevation in T_{core} by more than 1.0 °C is usually referred to as hyperthermia (Nybo et al., 2014) whereas the 0.6 °C raise in T_{rec} in the current study can be considered as heat stress. When the target T_{rec} was reached in HEAT and HEAT + CO_2, or an equivalent time is spent in the water bath in the CON trial participants were immediately transferred to the cycle ergometer mounted with an SRM power crank (SRM Science Road, Jülich, Germany) placed in a climatic chamber set to either 18 or 38 °C and 30% relative humidity.

After a 3-min resting period participants exercised for 5 min at 80 W, and 5 min at 130 W. Thereafter, the workload was increased by 30 W/min until exhaustion. Verbal encouragement was given toward the end of all trials. Maximal workloads reached in the exercise tests were calculated as $W_{max} = W_{compl} + W_{increm} \times (t/90)$ with W_{compl} being the last completed workload, W_{increm} the workload increment per exercise step and t the number of seconds in the not completed workload.

Experimental measures

Throughout each test $MCAv_{mean}$ was assessed as an estimate of CBF using transcranial Doppler ultrasonography (Doppler Box, DWL, Sipplingen, Germany). A 2-MHz probe prepared with ultrasound gel was adjusted over the temporal window to insonate the right MCA. To hold the probe in place throughout the exercise tests, the insonation probe was fastened to a headgear.

Cerebral and muscle tissue oxygenation (ScO_2, SmO_2) was monitored by near infra-red spectroscopy (NIRS, INVOS-5100c, Covidien, Mansfield, Massachusetts, USA). The sensor was placed on the right M. vastus lateralis and the left forehead.

T_{rec} was measured as a surrogate for T_{core}. A thermistor (MEAS 401AC, Measurement specialities, Dayton, Ohio, USA), was placed approximately 7 cm behind the internal anal sphincter. Additionally three skin temperature probes (MEAS 409AC) were placed on the forehead, the lower back and the right quadriceps. The average of the three locations was considered as mean skin temperature (T_{skin}).

MAP was recorded continuously and non-invasively via finger photoplethysmography (Nexfin, BMEYE B.V, Amsterdam, the Netherlands) and heart rate (HR) was assessed using a monitor belt (Cosmed Quark b2, Rome, Italy).

Participants breathed through a mouthpiece (Hans Rudolph, Shawnee, Kansas, USA) with their nose occluded. Ventilatory variables were measured breath-by-breath using a spirometer (Cosmed Quark CPET, Rome, Italy) consisting of a flow meter and fast responding gas analyzers. Prior to each experimental session the system was calibrated using a 3-L calibration syringe (Cosmed) and gas mixtures of known concentrations of O_2 and CO_2.

After the test all data points were averaged over the last 30 s of each workload. The highest average value for $\dot{V}O_2$ calculated over 30 s was adopted as $\dot{V}O_2max$.

Statistical analysis

Data were analyzed by two-way repeated measurements analysis of variance with exercise intensity (Rest, 80, 130, 160, 190, 220, 250, 280 W and Max) and condition (CON, HEAT and HEAT + CO_2) as main effects. Tukey's range test was applied for post-hoc analysis. Single comparisons were performed using a paired *t*-test with Bonferroni correction. A P value < 0.05 was considered statistically significant. All data are expressed as mean ± standard error of the mean unless otherwise indicated. Statistic analysis was performed using SAS Enterprise Guide (4.3, SAS Institute, Inc., Cary, North Carolina, USA).

Results

Exercise capacity

Both W_{max} and $\dot{V}O_2max$ where highest when conducted in CON, the values being 340 ± 10 W and 4.6 ± 0.1 L/min, respectively. Compared with CON W_{max} was decreased by 27.4 ± 7.6 W ($P < 0.01$) and 22.0 ± 7.8 W ($P < 0.05$) when conducted in HEAT and HEAT + CO_2, respectively. This corresponded to an individual relative change of 9.4 ± 2.8% and 7.3 ± 2.6%. The decrease in $\dot{V}O_2max$ was 0.4 ± 0.1 L/min (11.2 ± 4.1%, $P < 0.05$) and 0.4 ± L/min (10.9 ± 3.9%, $P < 0.05$). W_{max} and $\dot{V}O_2max$ in HEAT + CO_2 and HEAT were similar ($P = 0.50$ and $P = 0.96$, respectively).

Thermal and cardiovascular data

T_{rec} and T_{skin} data are shown in Fig. 1. Resting T_{rec} was 0.6 ± 0.0 °C higher ($P < 0.05$) in both, HEAT and HEAT + CO_2 compared with CON, but this difference was abolished ($P = 0.36$ and $P = 0.22$, respectively) at W_{max}. In agreement with T_{rec}, T_{skin} was higher ($P < 0.01$) in HEAT and HEAT + CO_2 than in CON at rest, but remained elevated throughout exercise ($P < 0.01$).

HR at rest and submaximal exercise was higher ($P < 0.05$) in HEAT and HEAT + CO_2 compared with CON, but reached similar maximal values ($P = 0.96$ and $P = 0.28$, respectively). MAP increased with exercise and was higher ($P < 0.05$) in the CON trial compared with HEAT and HEAT + CO_2 over the entire range of workloads.

Ventilatory variables

Analysis of the ventilatory response revealed an interaction between intensity and condition ($P < 0.01$). Ventilation (VE) in HEAT and HEAT + CO_2 was enhanced ($P < 0.01$) when compared with CON. Post-hoc analysis demonstrated that adding CO_2 to the inspiration tended ($P = 0.08$) to increase VE compared with the HEAT trial (Fig. 2(a)) and especially toward W_{max}. This difference was due to an increase in breathing frequency (42 ± 6

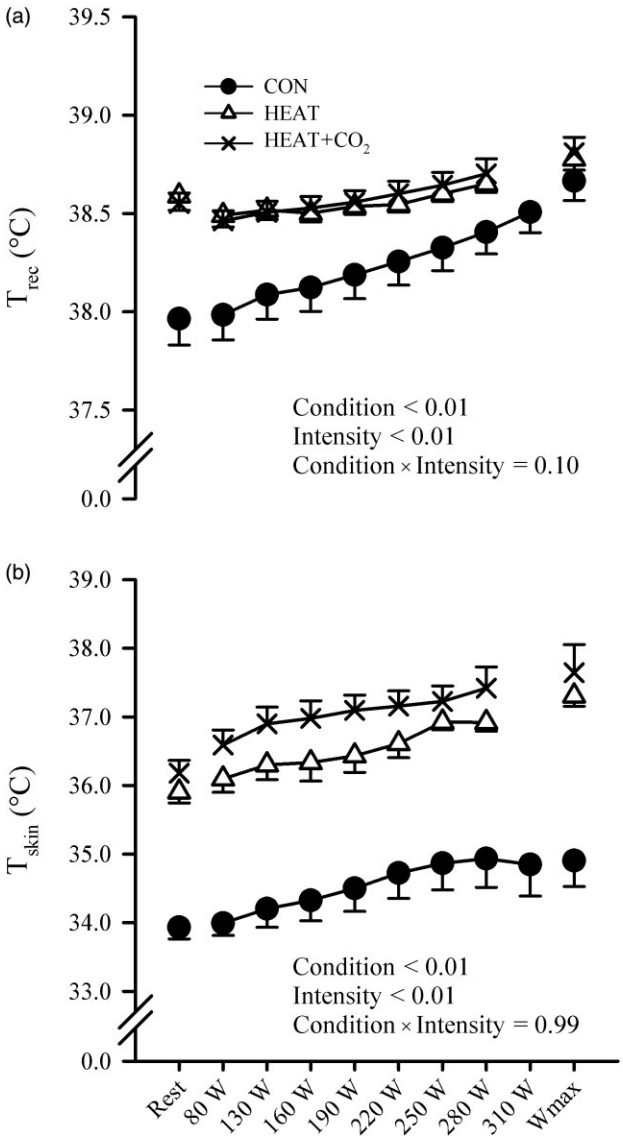

Fig. 1. (a) Rectal temperature (T_{rec}; °C) and (b) Mean skin temperature (T_{skin}; °C) at 18 °C (CON), 38 °C (HEAT) and 38 °C, but with administrated CO_2 (HEAT + CO_2). Measurements were completed at rest and throughout graded maximal exercise. Values are mean ± standard error of the mean. $N = 8$.

(HEAT) and 47 ± 5 breaths/min (HEAT + CO_2), $P < 0.01$) as tidal volume remained unchanged [3.1 ± 0.5 (HEAT) and 3.1 ± 0.4 L (HEAT + CO_2), $P = 0.50$].

$PaCO_2$ and $MCAv_{mean}$

Figure 2(b and c) illustrate $PaCO_2$ and $MCAv_{mean}$ for all exercise trials. Heat stress-induced hyperventilation led to a 1.3 ± 0.3 mmHg lower ($P < 0.05$) $PaCO_2$ in HEAT compared with CON during exercise. In the HEAT + CO_2 trial $PaCO_2$ was successfully prevented to decrease toward the end of exercise being 12.5 ± 4.4 and 13.7 ± 2.8% higher at W_{max} compared with CON and HEAT.

In HEAT $MCAv_{mean}$ was reduced ($P < 0.01$) compared with CON by −16.9 ± 1.7% at rest and by −18.3 ± 8.3%

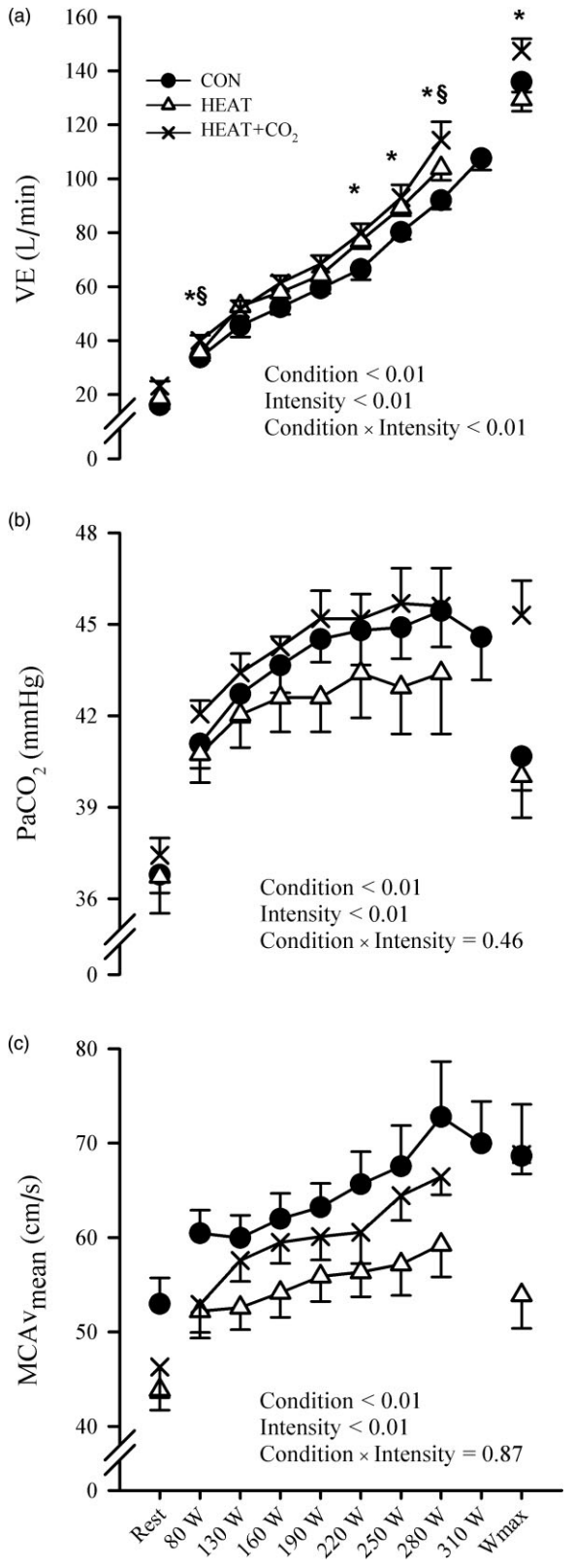

Fig. 2. (a) Ventilation (VE; L/min); (b) arterial PCO_2 ($PaCO_2$; mmHg); and (c) mean middle cerebral artery velocity ($MCAv_{mean}$; cm/s) at 18 °C (CON), 38 °C (HEAT) and 38 °C, but with administrated CO_2 (HEAT + CO_2). Measurements were completed at rest and throughout graded maximal exercise. Values are mean ± standard error of the mean. *$P < 0.05$ HEAT vs HEAT + CO_2, § < 0.05 CON vs HEAT + CO_2. $N = 8$.

at maximal effort. In line with $PaCO_2$, $MCAv_{mean}$ decreased in CON and HEAT but not in HEAT + CO_2. At rest $MCAv_{mean}$ of HEAT + CO_2 was reduced ($P < 0.01$) compared with CON. At W_{max} however, it reached a similar value.

A high association between the decline in $PaCO_2$ and $MCAv_{mean}$ was found. Pearson correlation between these two variables revealed a significant correlation ($P < 0.01$) with an R^2 value of 0.65.

Cerebral oxygenation

As indicated in Fig. 3, ScO_2 data were measured in CON ($N = 8$), HEAT ($N = 5$) and HEAT + CO_2 ($N = 6$). Overall, HEAT delta ScO_2 was lower ($P < 0.01$) compared with CON eliciting a 3.3 ± 0.8% lower value at maximal workload. Furthermore, and in line with an increased $MCAv_{mean}$ in HEAT + CO_2, an enhanced cerebral oxygenation at maximal effort was also apparent ($P < 0.05$) compared with HEAT. As expected and in contrast to ScO_2, CO_2 supplementation did not affect SmO_2. The exercise-induced reduction in SmO_2 was comparable in all three trials eliciting similar delta SmO_2 values at W_{max}.

Discussion

This study was designed to test the hypothesis that supplementing CO_2 to the inspiration during exercise in warm environments would prevent the heat-induced reduction in $MCAv_{mean}$. Although a reduced $MCAv_{mean}$ has previously been speculated to limit exercise capacity, we hypothesized that regardless of a concomitant improved cerebral oxygenation, a normalization of $MCAv_{mean}$ would not facilitate exercise performance. In agreement with our hypothesis, maintaining $PaCO_2$ during exercise in the heat prevented $MCAv_{mean}$ from becoming reduced. This, however, did not improve exercise performance arguing against a reduced $MCAv_{mean}$ being a major limiting factor for incremental exercise performance in heat-stressed humans.

Heat stress and ventilation

The reason for $MCAv_{mean}$ being reduced with heat stress is clearly linked to alterations in ventilation. Several studies have demonstrated that heat stress both at rest and during exercise is accompanied by an increase in pulmonary ventilation (Martin et al., 1979; Nybo & Nielsen, 2001b; White, 2006), although this is not always the case (Sawka et al., 1980). The reason for the minor divergent observations may be explained by discrepancies between experimental settings and the magnitude of heat stress. However, in resting humans, an elevation of T_{core} by ~1 °C is usually associated with hyperventilation, and during exercise, increases in pulmonary ventilation in the heat is positively correlated to

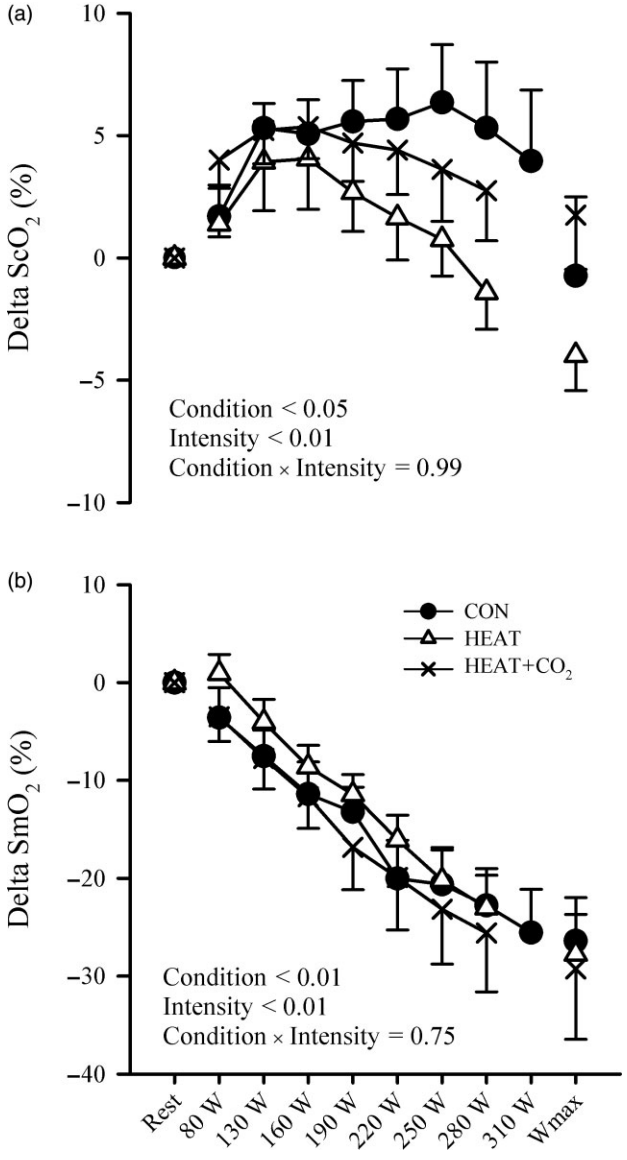

Fig. 3. (a) Changes in cerebral oxygenation (Delta ScO_2; %) and (b) Changes in muscle tissue oxygenation (Delta SmO_2; %) at 18 °C (CON), 38 °C (HEAT) and 38 °C, but with administrated CO_2 (HEAT + CO_2). Measurements were completed at rest and throughout graded maximal exercise. Values are mean ± standard error of the mean. For 2A $N = 8$ for CON, $N = 5$ for HEAT and $N = 6$ for HEAT + CO_2 and for 2B $N = 7$ for CON and HEAT and $N = 6$ for HEAT + CO_2.

T_{core} (White & Cabanac, 1996). Furthermore, Nybo and Nielsen (2001b) showed that heat stress superimposed to the normal exercise-induced elevation in esophageal temperature increased exercise ventilation by an additional 40%. Our study is well within this range considering the observed hyperventilation occurring with an elevated T_{rec} of 0.6 °C. The underlying mechanism(s) for heat stress-induced hyperventilation are still not entirely resolved. Possible candidates include, however, interactions of muscle metaboreflexes, blood gases and pH with temperature (Asmussen et al., 1965; Weil et al., 1972; Martin et al., 1979; Nybo & Nielsen, 2001b). Nonetheless, a direct thermal influence of temperature on the respiratory center is also possible (see also White, 2006, for complete review). Body tissue temperature is also speculated to be sensed by chemoreceptors located within the periphery at the carotid and/or aortic bodies and/or at central sites, possibly within the medulla oblongata (Mitchell et al., 1963; Mulkey et al., 2004). A physiological consequence of pulmonary ventilation being increased with an elevated body temperature despite $PaCO_2$ decreasing to values at which pulmonary ventilation is normally inhibited, is speculated to be a greater heat loss from the upper airways (Rapanos & Duffin, 1997). However, this and the potential underlying mechanisms need further research before clear-cut conclusions can be drawn.

$PaCO_2$ and $MCAv_{mean}$

As expected, heat stress-induced hyperventilation facilitated hypocapnia in the present study, which in turn likely also decreased $MCAv_{mean}$. Furthermore, by adding small amounts of CO_2 to the inspired air the drop in $PaCO_2$ could be prevented. This is in line with other studies in the heat were CBF was successfully manipulated by means of CO_2 supplementation during passive heating (Brothers et al., 2009; Nelson et al., 2011; Bain et al., 2013) and during submaximal exercise (Rasmussen et al., 2004). Accordingly, it seems safe to assume that the hyperventilation-induced drop in $PaCO_2$ is at least partially responsible for the decrement in $MCAv_{mean}$ in the heat. Indeed, it has previously been speculated that during passive heating, the reduction in $PaCO_2$ accounts for the entire decline in CBF (Nelson et al., 2011; Bain et al., 2013). However, during exercise in the heat, other possible mechanisms influencing CBF deserve being mentioned (see also Ogoh & Ainslie, 2009, for complete review). Especially the reduction in maximal $\dot{Q}$ and MAP are suggested to have a significant impact on CBF in heat-stressed humans (Nybo & Nielsen, 2001b). Furthermore, an increased cerebral sympathetic activity may lead to excessive vasoconstriction, which may also limit CBF (Querido & Sheel, 2007). However, since we were able to restore the decrease in $MCAv_{mean}$ at maximal workload by CO_2 supplementation it would seem that hyperventilation-induced hypercapnia is the main mechanism decreasing $MCAv_{mean}$ in heat-stressed individuals, at least during strenuous exercise.

Cerebral perfusion, oxygenation, and fatigue

Exercise performance is reduced when conducted with acute exposure to heat stress. While the involvement of peripheral factors (e.g., muscle, cardiovascular system) potentially contributing to premature fatigue have been studied extensively for the last decades (Rowell et al., 1966; Gonzalez-Alonso & Calbet, 2003;

Gonzalez-Alonso et al., 2008; Nybo, 2008; Nybo et al., 2014), more recent work suggest that the origin of heat-related fatigue is of predominantly central origin, and that especially, the reduced cerebral perfusion may be a prominent candidate as this may reduce cerebral oxygenation as well as heat removal (Nybo et al., 2002b). Indirect support that cerebral deoxygenation may affect exercise performance is based on studies where participants were switched to breathing a hyperoxic gas mixture immediately before the point of exhaustion in exercise tests conducted in hypoxia (Calbet et al., 2003; Amann et al., 2007; Subudhi et al., 2008). While this rapidly restores cerebral oxygenation and enables participants to continue exercising for several minutes, applying such study design obviously has its limitation as the administrated hyperoxia will undoubtedly also affect exercising muscle tissue oxygenation and not just that of the brain. By adding CO_2 instead of O_2, this limitation can be overcome as the CO_2 reactivity is greater in the cerebral compared with muscular vasculature (Ainslie et al., 2005). This is in line with the current study, where CO_2 supplementation enhanced cerebral, but not muscle tissue oxygenation, and indeed, skeletal muscle tissue oxygenation decreased similarly in all three trials. Thus, despite needs for augmented skin blood flow during exercise in the heat, blood flow, and thereby oxygenation of the skeletal muscle is prioritized, and thermoregulatory demands do not lead to a diversion of blood flow from active muscle (Nybo et al., 2014). As expected and in line with previous studies (Gonzalez-Alonso et al., 2004; Periard et al., 2013) cerebral oxygenation was impaired with exercise in the heat, which was however improved when $MCAv_{mean}$ was augmented in the CO_2 trial, and hence suggests that a reduced cerebral oxygenation is not the main cause for exercise performance being limited with heat stress. Although $MCAv_{mean}$ has previously not actively been manipulated in association with incremental exercise in the heat, $MCAv_{mean}$ has been restored in settings where $MCAv_{mean}$ is typically reduced. With exercise in hypoxia for example, the associated hyperventilation reduces $PaCO_2$, which in turn was thought to facilitate an even greater degree of cerebral deoxygenating and thereby also fatigue. However, three independent studies have recently demonstrated that normalization of $MCAv_{mean}$ to normoxic levels by means of CO_2 administration has no positive influence on exercise performance (Subudhi et al., 2011; Fan et al., 2012; Siebenmann et al., 2013), and the results from the present investigation are in line herewith. Flück et al. (2014) recently demonstrated that the age-induced decrease in cerebral perfusion is related to concomitant reductions in $PaCO_2$, but that normalization of $MCAv_{mean}$ to that of healthy young volunteers did not affect exercise performance, which is also in agreement with the current study. Thus, the present study is the first to mechanistically address the importance of a heat-induced reduction of $MCAv_{mean}$ on incremental exercise performance in the heat, and as previously reported in other settings, the diminished $MCAv_{mean}$ when exercising in a warm environment seems of only very limited importance for exercise performance.

In further support of our data, it has been demonstrated that the brain even at exhaustion possesses a large O_2 reserve, which is sufficient to compensate for reductions in blood flow and prevent the brain from severe deoxygenation, at least, when flow reductions are not excessive (Gonzalez-Alonso et al., 2004). The most prominent mechanism to prevent severe deoxygenation in both thermoneutral and hyperthermic conditions appears to be an elevated O_2 extraction, which seems capable of compensating for the observed decline in cerebral perfusion (Nybo et al., 2002a; Trangmar et al., 2014). It also seems that a reduction of about 50% in CBF would be required to impair cerebral O_2 in such a way that it affects exercise (Bain et al., 2014). This further strengthens the assumption that cerebral O_2 under hyperthermic conditions is not limiting incremental exercise performance.

Alternatively, the reduced cerebral perfusion could limit heat removal and thereby allow brain temperature to rise more rapidly and thereby induce premature fatigue (Nybo et al., 2002b). Our results suggest that normalizing $MCAv_{mean}$ does not facilitate brain cooling to such an extent where it affects incremental exercise performance. However, as brain temperature was not assessed in the current settings, we cannot rule out an influence of altered brain temperature on the results. Furthermore, the potential involvement of other, mainly peripheral factors, affecting exercise performance in the heat should be mentioned. Heat stress results in a distinct cardiovascular strain especially as more blood is diverted to the skin for thermoregulatory purposes (Sawka et al., 1979). The thermoregulatory demands associated to increasing skin blood flow and the metabolic demands to increase muscle blood flow bring cardiac pumping capacity to its limit, particularly during maximal intensity exercise. As a result, in a first step blood flow to the skin is reduced, likely resulting in a higher brain and body temperature and in a second step also blood flow to the exercising muscles and thus oxygen delivery is impaired, both leading to fatigue (Gonzalez-Alonso et al., 2008).

Taken together, increasing cerebral perfusion and oxygenation through CO_2-mediated vasodilation does not improve exercise performance in any of the yet tested conditions, and may suggest that although $MCAv_{mean}$ is reduced that the extent hereof is not sufficient to limit exercise capacity. Therefore, at least in heat-stressed individuals, the limitations to exercise must be found elsewhere, and either limitations within the cardiovascular system such as a maximally tasked cardiac output or an elevated hypothalamic temperature seem likely candidates.

Limitations to data interpretation

There are methodological limitations to the study, which deserve being discussed. First, although we successfully augmented $MCAv_{mean}$ and cerebral oxygenation in the CO_2 trial, the addition of CO_2 may by itself limit exercise performance and thereby perhaps offset any gains in cerebral function. Hypercapnia affects the blood acid–base balance in the blood, and the addition of CO_2 to the inspiration could hence be speculated to lower blood pH, which in turn could limit exercise performance. In studies from our research group applying a similar CO_2 administration regime as in the current study, no differences in capillary pH were observed when compared with a control trial (Flück et al., 2014). Nonetheless, in the current study, we did not measure blood pH and can therefore not rule out that an accumulated acidosis outweighed any potential benefits for increased $MCAv_{mean}$ or cerebral oxygenation. Second, the higher VE observed in the CO_2 trial will likely have resulted in the recruitment of additional skeletal muscle fibers within respiratory muscles, which in turn may have resulted in an increased perfusion of these fibers. This could have reduced blood flow to, for example, the exercising skeletal muscles and thereby offset a potential increase in exercise performance (Calbet et al., 2009). However, the hypothetic increase in respiratory muscle perfusion could also be diverted from non-exercise-involved organs, especially when considering the difficulties in redirecting blood flow during exercise (Lundby et al., 2008). A third limitation that should be discussed is the surrogate assessment of CBF by $MCAv_{mean}$. This approach is based on the assumption that the diameter of the insonated vessel remains unchanged across the evaluated conditions. Serrador et al. (2000) recently found no differences in vessel diameter with changes in $PaCO_2$. Nonetheless, we appreciate that a slight change in vessel diameter cannot be completely ruled out. However, a hypercapnia-induced vasodilation of the MCA would have led to an under- rather than overestimation of CBF, and thus our interpretation of the results remains valid. It should also be mentioned that the recoding of cerebral oxygenation (NIRS) in the heat proved challenging. We speculate that excessive sweating interfered with the probe and therefore caused missing values. In addition, elevations in skin blood flow may have introduced bias as this greatly influences the cerebral NIRS signal (Davis et al., 2006; Sorensen et al., 2012). An indication therefore is given by the fact that absolute ScO_2 values were approximately 10% higher in HEAT and HEAT + CO_2 compared with CON. However, for the analysis, only delta ScO_2 values were considered, which enable us to draw conclusions regardless of different resting values. Furthermore, cerebral oxygenation did show improvements in the CO_2 trial, suggesting that blood flow was indeed increased.

Perspectives

In conclusion, although CO_2 supplementation increases $MCAv_{mean}$ and cerebral oxygenation, this did not facilitate neither W_{max} nor $\dot{V}O_2max$. Hence, our results are first suggesting that a reduced $MCAv_{mean}$ is not a major cause for exercise performance being limited with heat stress. This is also in line with observations in normoxia and hypoxia. This, however, does not rule out that indeed, the CNS does limit exercise performance when conducted in the heat. The most likely candidate therefore could be brain temperature itself (Nybo & Rasmussen, 2007). If this is the case, then the present study at least suggests that normalization of $MCAv_{mean}$ does not simultaneously improve brain cooling to such an extent where it affects exercise performance. Furthermore, as brain temperature is stated to be approximately 0.2 °C higher than T_{core} (Nybo et al., 2002b), in the present study with a T_{rec} of ~39.0 °C, the brain temperature most likely did not reach critically high levels and rather cardiovascular limitations led to premature fatigue. However, to further understand the complexity of heat-induced centrally mediated fatigue, future studies should focus on brain temperature rather than oxygenation as a high hypothalamic temperature might play a crucial role in inhibiting motor activity and therefore lead to premature fatigue.

Key words: Brain blood flow, $PaCO_2$, exercise performance, cerebral oxygenation.

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Heat training increases exercise capacity in hot but not in temperate conditions: a mechanistic counter-balanced cross-over study

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[1]*Zürich Center for Integrative Human Physiology, Institute of Physiology, University of Zürich, Zürich, Switzerland;* [2]*Exercise Physiology, Institute of Human Movement Sciences, ETH Zürich, Zürich, Switzerland;* [3]*Intensive Care Unit, University Hospital of Zürich, Zürich, Switzerland;* [4]*Food and Nutrition and Sport Science, Gothenburg University, Gothenburg, Sweden*

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Keiser S, Flück D, Hüppin F, Stravs A, Hilty MP, Lundby C. Heat training increases exercise capacity in hot but not in temperate conditions: a mechanistic counter-balanced cross-over study. *Am J Physiol Heart Circ Physiol* 309: H750–H761, 2015. First published July 1, 2015; doi:10.1152/ajpheart.00138.2015.—The aim was to determine the mechanisms facilitating exercise performance in hot conditions following heat training. In a counter-balanced order, seven males ($\dot{V}O_{2max}$ 61.2 ± 4.4 ml·min^{-1}·kg^{-1}) were assigned to either 10 days of 90-min exercise training in 18 or 38°C ambient temperature (30% relative humidity) applying a cross-over design. Participants were tested for $\dot{V}O_{2max}$ and 30-min time trial performance in 18 (T18) and 38°C (T38) before and after training. Blood volume parameters, sweat output, cardiac output ($\dot{Q}$), cerebral perfusion (i.e., middle cerebral artery velocity [$MCAv_{mean}$]), and other variables were determined. Before one set of exercise tests in T38, blood volume was acutely expanded by 538 ± 16 ml with an albumin solution ($T38_A$) to determine the role of acclimatization induced hypervolemia on exercise performance. We furthermore hypothesized that heat training would restore $MCAv_{mean}$ and thereby limit centrally mediated fatigue. $\dot{V}O_{2max}$ and time trial performance were equally reduced in T38 and $T38_A$ (7.2 ± 1.6 and 9.3 ± 2.5% for $\dot{V}O_{2max}$; 12.8 ± 2.8 and 12.9 ± 2.8% for time trial). Following heat training both were increased in T38 (9.6 ± 2.1 and 10.4 ± 3.1%, respectively), whereas both $\dot{V}O_{2max}$ and time trial performance remained unchanged in T18. As expected, heat training augmented plasma volume (6 ± 2%) and mean sweat output (26 ± 6%), whereas sweat [Na^+] became reduced by 19 ± 7%. In T38 $\dot{Q}_{max}$ remained unchanged before (21.3 ± 0.6 l/min) to after (21.7 ± 0.5 l/min) training, whereas $MCAv_{mean}$ was increased by 13 ± 10%. However, none of the observed adaptations correlated with the concomitant observed changes in exercise performance.

hyperthermia; blood volume; performance; training; temperature

NEW & NOTEWORTHY

In this study, we demonstrate that 10 days of heat training facilitates exercise performance in the heat but not in temperate conditions. Training-induced changes in physiological parameters, which have previously been suggested to facilitate such responses, were, however, not associated with the observed gains in exercise performance.

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ENDURANCE EXERCISE PERFORMANCE is impaired with acute exposure to warm environments but gradually recovers, at least partially, with heat acclimatization (naturally occurring exposures) and/or acclimation (experimentally induced heat adaptation) (21, 23, 38). Physiological adjustments to such heat acclimation have been investigated for centuries (5, 29, 41), and one phenotypic adaptation proposed to facilitate exercise capacity with both acclimatization and acclimation is the hyperthermia-induced expansion of plasma volume (PV) (21, 23, 39). Seemingly, such adaptation could to some extent explain several improvements such as an increased sweat output (24, 40) and maximal cardiac output ($\dot{Q}$) (21) and a reduced submaximal heart rate (HR) (24, 40, 49) and submaximal core temperature (T_{core}) (40), which could potentially all contribute to enhanced exercise performance. However, acute expansion of PV (e.g., with albumin) has failed to reduce HR and T_{core} during submaximal walking (37) and cycling (48) in humans exposed to heat stress. Likewise, PV expansion did not modify T_{core}, HR, and exercise performance in the heat (48). However, due to an increase in PV, heat acclimation might also diminish hyperthermia-induced reduction in cerebral perfusion that may accelerate centrally mediated fatigue (25, 35). An increase in cerebral perfusion following heat training could also be speculated to improve cooling of the brain as proposed by Nybo et al. (28). Nevertheless, we did not observe any $\dot{V}O_{2max}$ improvement despite restoring middle cerebral artery velocity ($MCAv_{mean}$) by administering small volumes of inspired CO_2 during exercise in hot conditions (17). However, the influence of altering cerebral pH via CO_2 inhalation on exercise performance, independent of cerebral perfusion, remains uncertain. One aim of the current investigation was to determine the relevance of heat acclimation-induced changes in PV and $MCAv_{mean}$ for facilitating exercise performance in the heat.

Further adaptive responses to repeated heat exposure, which may favor exercise performance in the heat, are suggested to involve a reduced loss of electrolytes in sweat (20, 24). This might be favorable for hour-long submaximal exercise tasks, although it appears of minor relevance for near maximal or maximal intensity exercise challenges such as 5,000 m running.

Heat acclimation has also been reported to augment exercise capacity in conditions comprising normal (21–21.5°C) (22, 38)

and cool (13°C) (21) ambient temperatures. Of note, only the latter study included a control group. Lorenzo et al. (21) showed that 10 days of heat acclimation improves $\dot{V}O_{2max}$ and time trial performance when tested in 13°C ambient temperature by 5% and 6%, respectively, in well-trained subjects. Yet, the enhanced effect on exercise performance should be taken with caution since the heat training was conducted at a relative higher exercise intensity when compared with control training. Although the difference in exercise intensity is estimated to be within ~20%, and below the anaerobic threshold in both trials, it cannot be ruled out that at least some of the observed effects were the result of more intense training. Accordingly, it was shown that heat training conducted in competitive cyclists and appropriate control subjects led to no further improvement in normothermic $\dot{V}O_{2max}$ or outdoor Time Trial performance (16). One further aim with the current investigation was to test whether heat training facilitates exercise performance in temperate conditions.

Therefore, with the current study we sought to determine physiological mechanisms leading to improved exercise capacity in the heat following heat acclimation. We hypothesized that the enhanced exercise performance would be primarily attributed to the increase in $MCAv_{mean}$ rather than an expansion of PV. To test this hypothesis, PV was acutely expanded by 15% (corresponding to the expected heat acclimation increase in PV) before exercise conducted with acute exposure to 38°C and $MCAv_{mean}$ and cardiovascular parameters were assessed. Furthermore, $MCAv_{mean}$ and cardiovascular parameters were also assessed after 10 days of heat training. A further aim was to test whether heat acclimation increases exercise capacity when performed in 18°C ambient temperature. This was tested by a randomized and counterbalanced cross-over design. We hypothesized that heat training, if conducted at a relative similar cardiovascular strain as in temperate conditions, would not enhance exercise performance in 18°C ambient temperature.

METHODS

Participants. Eight well-trained males (24 ± 2 yr, 74 ± 3 kg, 182 ± 6 cm, $\dot{V}O_{2max}$ 61.2 ± 4.4 ml·min^{-1}·kg^{-1}, BMI 22.3 ± 1.7, means ± SD) who regularly trained >1 h/day for 3–5 days/wk were recruited to participate in the study. Before participation oral and written informed consent was obtained from each participant. All participants refrained from exercise for 24 h and alcohol and caffeine for 12 h before the experimental tests and fulfilled the inclusion criterion of a $\dot{V}O_{2max}$ >55 ml·min^{-1}·kg^{-1}. In addition, they were instructed not to donate blood and to avoid ingestion of nonprescription drugs for the entire duration of the multiple study visits. All experimental protocols and procedures were approved by the ethical committee of the Swiss Federal Institute of Technology Zürich (EK 2013-N-23) and conformed to the Declaration of Helsinki. A limited set of data collected as part of this study has been published elsewhere (17).

Study design. A randomized and counter-balanced cross-over design was applied, and, therefore, participants completed both the heat and the control trainings (Fig. 1).

All participants completed a preliminary maximal incremental exercise and 30-min Time Trial test in a temperate environment (18°C) to become familiar with the experimental set-up. Thereafter, participants completed a battery of physiological and performance tests in two environmental conditions (18°C and 38°C, both at 30% relative humidity), then completed either a heat (38°C) or a control training (18°C) period of 10 days where after the tests were repeated. The first training block was followed by a 3-mo washout period after which the second block started. During this washout period, participants continued with their regular training and avoided any sojourn to

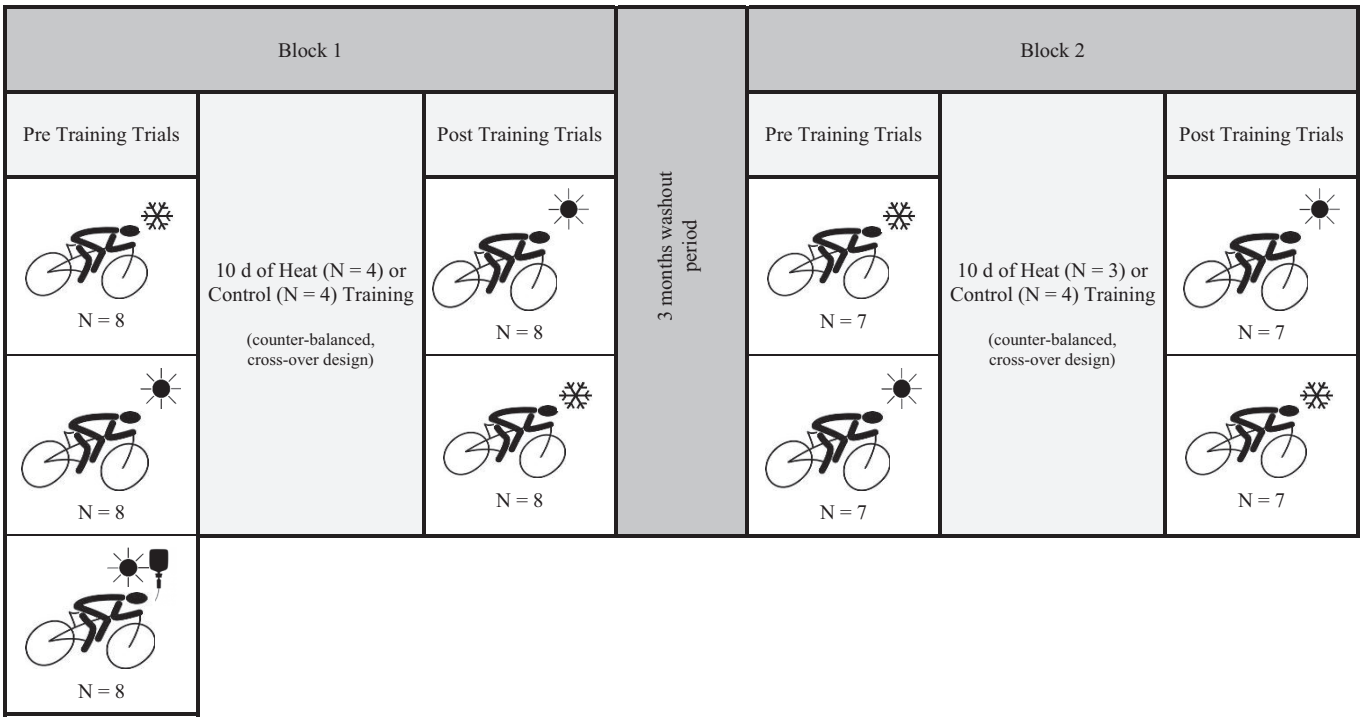

Fig. 1. Schematic illustration of the study design. The albumin trial was performed in the pretraining trials of *Block 1*. The data set for the experimental trial (Heat) consisted of the pre- and postheat training values from *Blocks 1* and *2*, and the data set for the control trial (Control) consisted of the pre- and postcontrol training values also from *Blocks 1* and *2*.

conditions above 25°C. The second block was identical to the first except that participants who first trained in cool conditions now trained in the heat and vice versa. Additionally, only in the first block and before the training period an additional experimental trial in the heat ($T38_A$) was conducted in which albumin was infused to expand the PV by 15%. This $T38_A$ trial was conducted once in all participants independently of their subsequent training group assignment and was afterwards compared with the baseline T18 and the T38 trials of the first block.

The performance tests included a maximal incremental exercise test ($\dot{V}O_{2max}$ test) followed by a Time Trial test after a 1-h resting period. To restore fluid compartments and energy stores, ~45 min before the $\dot{V}O_{2max}$ test participants drank 0.4 l of a carbohydrate-electrolyte solution (Isostar) and 0.6 l during the resting period between the $\dot{V}O_{2max}$ and Time Trial test. During the exercise tests, participants were not allowed to drink. The order of the training condition was randomized and counter-balanced, and all test sessions were separated by at least 48 h. After acclimation, studies were completed within 4 to 5 days of completion of the training period.

All experiments took place during the cool seasons of fall and winter, when participants were naturally unacclimatized to heat. For the entire duration of the study, the temperatures in Switzerland did not exceed 15°C. For the measurements conducted in the first block the average outdoor air temperature was 14.6°C and for the second block 4.3°C. The temperature inside where the experiments were conducted was held as constant as possible ranging from 37°C to 39°C in the T38 trials and from 17°C to 19.5°C in the cold trials.

Exercise performance tests. Before each exercise test ($\dot{V}O_{2max}$ and Time Trial), participants were immersed into a whole body water bath for 21 ± 5.8 min (means ± SD). The aim herewith was to manipulate rectal (core) temperature without performing exercise. Although before the T18 testing the water temperature was thermoneutral (~34°C), it was ~42°C before the T38 and $T38_A$ trials. This allowed increasing ($P < 0.05$) participants' rectal temperature (T_{rec}) by 0.8 ± 0.5°C (means ± SD) in the T38 and the $T38_A$ trial, whereas in the T18 trial T_{rec} remained unaffected. When target T_{rec} was reached in T38 and $T38_A$, or an equivalent time spent in the water bath in the T18 trial, participants were immediately transferred to the cycle ergometer (Monark Ergomedic 839 E, Vansbro, Sweden) mounted with a triathlon handlebar and a SRM power crank (SRM Science Road, Jülich, Germany) placed in a climatic chamber set to either 18°C or 38°C and 30% relative humidity.$\dot{V}O_{2max}$. After a 3-min resting period, where resting values were obtained, participants exercised for 5 min at 80 and 5 min at 130 W. Thereafter, workload was increased by 30 W every 90 s until exhaustion. Verbal encouragement was given toward the end of all trials. Maximal workloads reached in the exercise tests were calculated as $W_{max} = W_{compl} + W_{increm} * (t/90)$, with W_{compl} being the last completed workload, W_{increm} the workload increment per exercise step, and *t* the number of seconds in the not completed workload.

Time trial. After a 1-h resting period and 20-min water immersion, participants performed a Time Trial test. This recovery time has been demonstrated adequate to prevent any bias in subsequent aerobic performance tests (19). After a 3-min resting period, where again resting values were obtained, and a subsequent 5-min warm-up period at a self-selected workload, participants provided their maximal effort for 30 min. Average power output during 30 min (P_{avg}) was the performance measured. During the test, participants were allowed to modify power output as often as needed and were aware of the current power output and total time elapsed.

Plasma volume expansion. Participants were positioned on a bed and prepared with a 18-gauge catheter placed in an antecubital vein. Preceding the water submersion, a volume corresponding to 15% (538 ± 16 ml) of the participants' PV was then infused in form of 20% human albumin (albumin CSL 20%; CSL Behring, Bern, Switzerland). Such albumin infusion has been reported to expand and maintain an individuals' PV for >6 h (9). Blood pressure and HR were continuously monitored to ensure participants' well-being. After completion of the expansion and as soon as participants felt ready, the exercise performance tests were initiated as described above.

Training intervention. The training loads were set corresponding to the HR elicited at 50% of T18 and T38 $\dot{V}O_{2max}$ with the intent to assure the same relative cardiovascular strain in both conditions. Each training session lasted 90 min and was conducted on 10 consecutive days, which is considered sufficient to trigger substantial heat acclimation. To minimize dehydration, every 30 min participants were provided with 0.5 l of a carbohydrate and electrolyte enriched drink (Isostar) independent of whether they trained in hot or temperate conditions.

Experimental measures. Throughout each test T_{rec} was measured as a surrogate for T_{core}. A flexible rectal probe (YSI M401AC; Advanced Industrial Systems, Louisville, KY) was self-inserted by the participant ~7 cm behind the anal sphincter. Additionally, three skin temperature probes (YSI M409AC; Advanced Industrial Systems, Louisville, KY) were placed on the forehead, the lower back, and the right quadriceps. The average of the three locations was considered as mean skin temperature (T_{skin}). HR was assessed using a monitor belt (Cosmed, Rome, Italy). and mean arterial pressure (MAP) was measured noninvasively by means of finger photoplethysmography (Nexfin, BMEYE B.V, Amsterdam, Netherlands).

During the $\dot{V}O_{2max}$ test participants breathed through a mouthpiece (Hans Rudolph, Shawnee, KS) with their noses occluded wearing a nose clip. Ventilatory variables were measured breath by breath using an indirect calorimeter (Cosmed Quark CPET, Rome, Italy) consisting of a flow meter and fast responding gas analysers. Before each experimental session, the system was calibrated using a 3-l calibration syringe (Cosmed, Rome, Italy) and gas mixtures of known concentrations of O_2 and CO_2. After the test all data points were averaged over the last 30 s of each workload. The highest average value for $\dot{V}O_2$ calculated over 30 s was taken as $\dot{V}O_{2max}$.

During the $\dot{V}O_{2max}$ test $\dot{Q}$ and stroke volume (SV) were assessed at 80 and 130 W and then every 3 min with the Innocor M400 device (Innovision, Glamsbjerg, Denmark), which is based on an inert gas rebreathing technique previously described elsewhere (42). When a measurement is initiated, participants are switched from breathing room air to the closed circuit and breathe a known gas mixture for 3 breaths, which concentration allows making predictions about $\dot{Q}$. Measurements are based on the assumption that pulmonary uptake of blood soluble testing gas is proportional to pulmonary blood flow, which in turn can be considered equal to cardiac output. The highest obtained value was defined as $\dot{Q}$ and SV_{max}, respectively.

$MCAv_{mean}$ was assessed as an estimate of cerebral blood flow (CBF) using transcranial Doppler ultrasonography (Doppler Box, DWL, Singen, Germany). A 2-MHz probe prepared with ultrasound gel was adjusted over the temporal window to insonate the right MCA and was held in place with a snug-fitting headgear. Great care was taken that $MCAv_{mean}$ was always assessed at the same angle, position (pictures were taken for this), and depth. Cerebral tissue oxygenation (Sco_2) was monitored by near infra-red spectroscopy (NIRS; INVOS-5100c; Covidien, Minneapolis, MN) on the left forehead. Furthermore, $MCAv_{mean}$ and Sco_2 are always presented as delta values (%baseline) to draw conclusions independently of different resting values.

Dry, nude body weight was determined at the beginning and at the end of each exercise test and training session by a precision weighing balance to the nearest 0.1 kg (Kern MPB300K100; Balingen, Germany). After correcting for fluid intake and time, the weight difference before and after the tests or trainings was considered as sweat output.

Sweat samples were obtained during the training sessions on *Days 1* and *10* after 30, 60, and 90 min of exercise. First the skin area between the scapulae was cleared with ultra-pure water (Milli Q, 18.2 MΩ ionic purity). A cotton tissue was then placed on the same area and fixed with tape. After 5 min, the cotton tissue was removed and

squeezed and transferred into a syringe (Pico 50; Radiometer, Brønshøj, Denmark). The obtained sweat was then analyzed in duplicate for Cl^-, K^+, Ca^{2+}, and Na^+ concentration ([Cl^-], [K^+], [Ca^{2+}], [Na^+]) by an automated hemoximeter (ABL800; Radiometer, Copenhagen, Denmark).

PV, red blood cell volume (RBCV), and total blood volume (BV) was estimated with a CO rebreathing method introduced by Burge and Skinner (4) but included small modifications. Briefly, participants were positioned on a bed with elevated legs to facilitate venous return (18). Then, a 18-gauge catheter was placed in an antecubital vein and participants were asked to drink 0.5 l of water. This was followed by a 4-min period were participants breathed 100% oxygen. Although participants were still connected to the oxygen, a blood sample was taken and analyzed in quadruplicate for the fraction of carboxyhemoglobin (HbCO) and hemoglobin concentration ([Hb]) on a hemoximeter (ABL800; Radiometer, Copenhagen, Denmark) and for hematocrit (Hct) by the micro-method (4 min at 13,500 rpm). Immediately afterward, participants were switched to a closed rebreathing circuit and breathed 1.5 ml/kg of 99.997% chemically pure CO (CO N47; Air Liquide, Pullach, Germany) for 10 min. After these 10 min, another blood sample was taken and analyzed the same way as the first one. Finally, BV parameters could be derived from these variables (4). In this study the coefficient of variance, expressed as percent typical error (15), was 1.8% for Hb_{mass} and 3.8% for PV.

Statistical analysis. Single differences between the T18, T38, and the $T38_A$ trial were evaluated with a one-way ANOVA for repeated measures, whereas a repeated two-way ANOVA design with exercise intensity (Rest, 80 W, 130 W, 160 W, 190 W, 220 W, 250 W, 280 W, Max) and condition (T18, T3, $T38_A$) as main effects was applied for the parameters recorded at different intensities. To detect changes from pre to post again, a two-way ANOVA for repeated measures with two times (pre and post) and two interventions (experimental and control) was performed. The parameters recorded at different intensities were analyzed with a three-way ANOVA with the additional main effect of intensity (Rest, 80 W, 130 W, 160 W, 190 W, 220 W, 250 W, 280 W, Max). Tukey's range test was applied for post hoc analysis after a significant F main effect and interaction. Where appropriate, single comparisons were made using a paired *t*-test. Bivariate associations were determined by Pearson's correlation coefficients where $r = 0.1$ represents a small, $r = 0.3$ a moderate, and $r = 0.5$ a large correlation. A P value <0.05 was considered statistically significant. Effect-sizes were described using Cohen's d (with $d \leq 0.2$ representing a trivial difference; 0.2–0.5, a small difference; 0.5–0.8 a moderate difference; and >0.8 a large difference). Data are expressed as means ± SE unless otherwise indicated. Statistical analysis was performed using SAS Enterprise Guide (4.3; SAS Institute, Cary, NC).

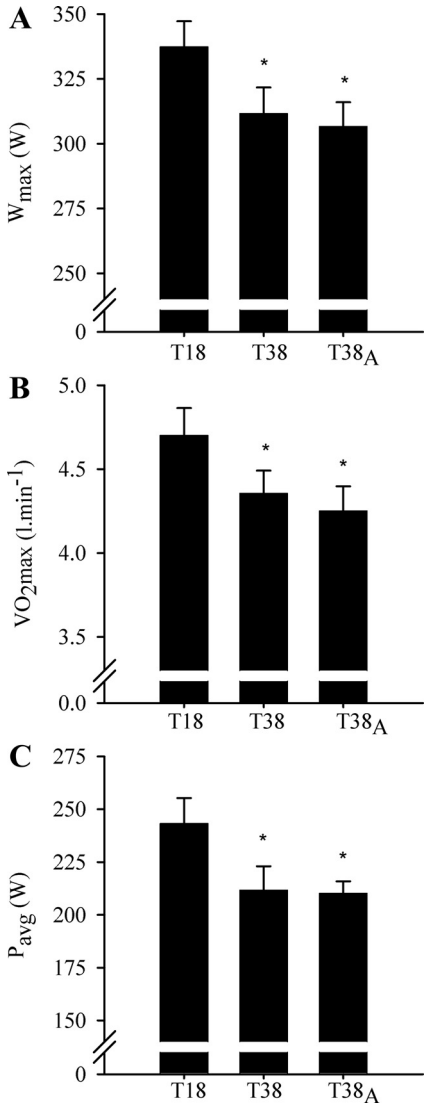

Fig. 2. Maximal achieved workload (W_{max}; in W) (*A*), oxygen uptake ($\dot{V}O_{2max}$; in l/min) (*B*), and average power output (P_{avg}; in W) (*C*) in temperate conditions (30-min time trial performance in 18°C, or T18), hot conditions (30-min time trial performance in 38°C, or T38), and hot conditions with prior albumin infusion (30-min time trial performance in 38°C with an albumin solution, or $T38_A$) (before the training intervention). Values are means ± SE. *$P < 0.05$ vs. T18; $N = 8$.

RESULTS

One volunteer withdrew from the study after completing the first block due to personal reasons. Therefore, the experimental group consisted of seven participants. The $T38_A$ trial (conducted before the exercise training intervention), however, was completed by all eight volunteers.

Exercise performance with acute heat exposure following plasma volume expansion. W_{max} (Fig. 2*A*), $\dot{V}O_{2max}$ (Fig. 2*B*), and P_{avg} (Fig. 2*C*) were highest when conducted during the T18 trial. When compared with T18, W_{max} was decreased by 7.6 ± 1.5 and 9.0 ± 2.1% (both $P < 0.01$) in T38 and $T38_A$. The corresponding reduction in $\dot{V}O_{2max}$ was 7.2 ± 1.6 and 9.3 ± 2.5% ($P < 0.05$), respectively. P_{avg} was reduced by 12.8 ± 2.8 and 12.9 ± 2.8% (both $P < 0.05$) in T38 and $T38_A$. W_{max}, $\dot{V}O_{2max}$, and P_{avg} during the T38 and $T38_A$ trials were similar ($P > 0.99$, $P = 0.9$, and $P > 0.99$, respectively).

Figure 3 illustrates HR, SV, and $\dot{Q}$ assessed during the $\dot{V}O_{2max}$ test. Overall HR was higher ($P < 0.01$) during the T38 compared with the T18 trial. HR was decreased ($P < 0.01$) in the $T38_A$ trial compared with the T38 trial but still elevated ($P < 0.01$) compared with the T18 trial. Overall, albumin infusion led to a higher ($P < 0.01$) SV and $\dot{Q}$ compared with the T18 and T38 trial. Furthermore, overall SV but not $\dot{Q}$ was decreased ($P < 0.01$ and $P = 0.97$, respectively) when conducted during the T38 trial compared with the T18 trial. The same applied for SV_{max} and $\dot{Q}_{max}$, which were enhanced ($P < 0.01$) with albumin infusion compared with the T18 and T38 trial but did not differ ($P = 0.53$, $P = 0.78$) between T18 and T38.

Albumin infusion did not alter VE ($P = 0.38$) in the $T38_A$ $\dot{V}O_{2max}$ test when compared with the T38 trial. MAP was

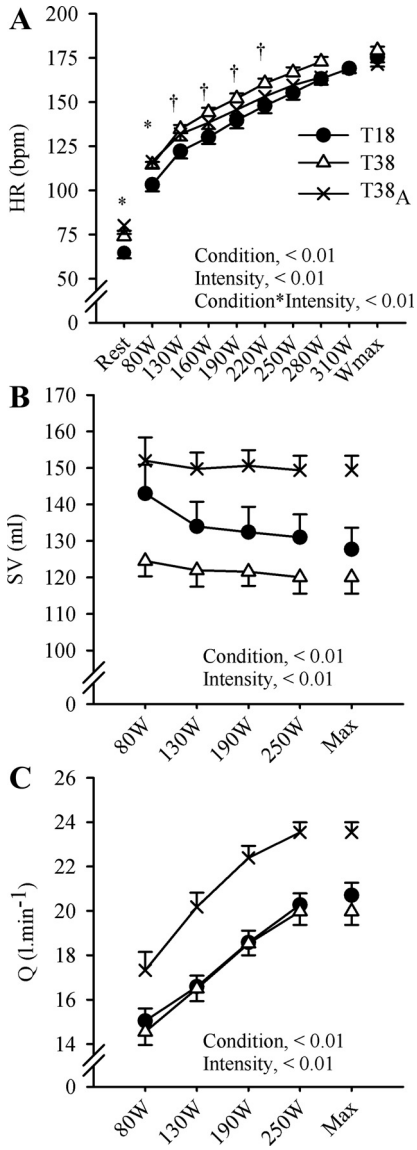

Fig. 3. Heart rate [HR; in beats/min (bpm)] (*A*), stroke volume (SV; in ml) (*B*), and cardiac output ($\dot{Q}$; in l/min) (*C*) in temperate conditions (T18; ●), hot conditions (T38; △), and hot conditions with prior albumin infusion ($T38_A$; ×) (before the training intervention). Values are means ± SE. *$P < 0.05$, T18 vs. $T38_A$; †$P < 0.05$, T18 vs. T38; $N = 8$.

increased ($P < 0.01$) in the $T38_A$ trial compared with the T38 trial but was still reduced ($P < 0.01$) compared with the T18 trial. $MCAv_{mean}$ was also reduced in the T38 trial compared with the T18 trial. With albumin infusion, however, $MCAv_{mean}$ was restored to the same level as in the T18 trials throughout the entire exercise test ($P = 0.96$).

Detailed thermoregulatory parameters of all three trials are displayed in Table 1. Briefly, due to the passively induced hyperthermia before exercise, resting T_{rec} and T_{skin} reached higher ($P < 0.05$ for T_{rec} and $P < 0.01$ for T_{skin}) levels during the T38 and $T38_A$ trial than in the T18 trial. Furthermore, during the T38 and $T38_A$, trial maximal T_{rec} and T_{skin} were higher ($P < 0.05$) compared with the T18 trial in the Time Trial but not in the $\dot{V}O_{2max}$ test for T_{rec} ($P = 0.53$ and $P = 0.37$, respectively). Albumin infusion did not lead to a reduction ($P > 0.99$, $P = 0.15$) in maximal T_{rec} and T_{skin} compared with the T38 trial.

Effects of heat training on exercise performance when conducted in 18° and 38°C. Figure 4 summarizes the mean performance responses for each test and for both groups. Before heat training, a hyperthermia-induced reduction in W_{max} (8.5 ± 2.2%, $P < 0.01$), $\dot{V}O_{2max}$ (4.1 ± 1.4%, $P < 0.05$), and P_{avg} (13.1 ± 2.9%, $P < 0.01$) was apparent in the T38 trial compared with the T18 trial. There was a significant main effect of time and time*intervention interaction from pre to post when conducted at 38°C for W_{max}, $\dot{V}O_{2max}$, and Time Trial P_{avg}. In the experimental group, W_{max} increased by 7.9 ± 1.7% (311 ± 13 vs. 335 ± 12 W, $P < 0.01$, d = 0.74), $\dot{V}O_{2max}$ by 9.6 ± 2.1% (4.3 ± 0.2 vs. 4.8 ± 9.2 l/min, $P < 0.05$, d > 0.80), and P_{avg} by 10.4 ± 3.1% (208 ± 12 vs. 228 ± 11 W, $P < 0.01$, d = 0.63). In contrast neither were elevated compared with preheat training when tested in T18, since there was no main effect of time ($P = 0.19$ for W_{max}, $P = 0.11$ for $\dot{V}O_{2max}$, and $P =$ for 0.32 for P_{avg}) or intervention ($P = 0.61$ for W_{max}, $P = 0.22$ for $\dot{V}O_{2max}$, and $P = 0.27$ for P_{avg}), and no interaction of the two ($P = 0.52$ for W_{max}, $P = 0.57$ for $\dot{V}O_{2max}$, and $P = 0.64$ for P_{avg}) from pre to post (340 ± 13 vs. 350 ± 10 W, d = 0.32 for W_{max}, 4.5 ± 0.2 vs. 4.7 ± 0.1 l/min, d = 0.43 for $\dot{V}O_{2max}$ and 240 ± 15 vs. 246 ± 14 W, d = 0.16 for P_{avg}).

Blood volume parameters. The expansion of PV with albumin by 15% corresponded to an increase ($P < 0.01$) in PV of 538 ± 16 ml. This was more ($P < 0.05$) than the corresponding increase induced by heat training (6 ± 2%, 201 ± 88 ml). Nonetheless, the results of the CO rebreathing revealed a significant time*intervention interaction ($P < 0.05$), due to an increase in PV for the experimental group (+6 ± 2% from 3,370 ± 115 to 3,571 ± 169 ml; $P < 0.05$; d = 0.53) but an unaltered PV for the control group (3,524 ± 128 vs. 3,467 ± 135 ml; $P = 0.80$; d = 0.15).

Heat training did not affect RBCV (2,563 ± 113 vs. 2,523 ± 111 ml; d = 0.19), and also in the control group no differences in RBCV (2,546 ± 98 vs. 2,511 ± 95 ml; d = 0.13) were observed, since there was no main effect of time ($P = 0.22$) or intervention ($P = 0.93$), and no interaction ($P = 0.95$) of the two from pre to post.

Although there was a significant time*intervention interaction ($P < 0.05$) with no main effect for time ($P = 0.59$) and intervention ($P = 0.68$) for total BV, the post hoc analysis for the experimental group revealed no significantly increased total BV after heat training (5,916 ± 210 vs. 6,111 ± 257 ml; $P = 0.12$; d = 0.31). Also in the control group, BV remained unchanged from pre- to posttraining (6,089 ± 200 vs. 5,949 ± 187 ml; $P = 0.25$; d = 0.26).

Effects of heat training on cardiovascular and thermoregulatory parameters. Detailed cardiovascular and thermoregulatory responses of all exercise tests are provided in Table 2. Briefly, in both the experimental and the control group, training did not lead to an altered $\dot{Q}_{max}$ or SV_{max}. T_{rec} in the T38 trials was always elevated to the same initial temperature by water immersion, and therefore resting T_{rec} did not differ from pre- to posttraining. In contrast, we did not control for T_{rec} in the T18 trials, and therefore participants' resting T_{rec} varied by ~0.25 ± 0.04°C (main effect of time $P < 0.05$). However, no significant time*intervention interaction could be found due to similar changes in the exercise and the control group. The same

Table 1. *Thermoregulatory responses to exercise ($\dot{V}O_{2max}$ and time trial tests) in temperate conditions (T18), hot conditions (T38), and hot conditions with prior albumin infusion ($T38_A$)*

	$\dot{V}O_{2max}$ Test			Time Trial Test		
	T18	T38	$T38_A$	T18	T38	$T38_A$
Resting T_{rec}, °C	38.0 ± 0.1†	38.8 ± 0.2*	38.9 ± 0.1*	37.9 ± 0.1††	38.8 ± 0.2**	39.0 ± 0.1**
Maximal T_{rec}, °C	38.5 ± 0.2	38.8 ± 0.1	38.8 ± 0.1	39.2 ± 0.1††	39.6 ± 0.2*	39.6 ± 0.1*
Resting T_{skin}, °C	34.0 ± 0.2††	37.0 ± 0.3†*	37.8 ± 0.2**	32.6 ± 0.5††	36.9 ± 0.4**	37.4 ± 0.8**
Maximal T_{skin}, °C	36.8 ± 0.5†	37.7 ± 0.3*	38.2 ± 0.3*	36.4 ± 0.4†	38.1 ± 0.3*	38.2 ± 0.3*

Values are means ± SE; $n = 8$. Resting T_{rec}, resting rectal temperature; maximal T_{rec}, maximal rectal temperature; resting T_{skin}, resting mean skin temperature; maximal T_{skin}, maximal mean skin temperature; T18, $\dot{V}O_{2max}$ or 30-min time trial performance in 18°C; T38, $\dot{V}O_{2max}$ or 30-min time trial performance in 38°C; $T38_A$, $\dot{V}O_{2max}$ or 30-min time trial performance in 38°C with an albumin solution. *$P < 0.05$ and **$P < 0.01$ vs. T18; †$P < 0.05$ and ††$P < 0.01$ vs. $T38_A$.

was true for maximal T_{rec} but only in the Time Trial test. Statistical analysis revealed no other significant training-induced changes in any of the measured parameters.

Effects of heat training on ventilation, mean arterial pressure, and cerebral perfusion. Exposure to heat (T38) increased ($P < 0.05$) ventilation (VE) compared with the T18 trial (Fig. 5*A*). For both the T18 and the T38 trial, a significant main effect for time and intensity was found; however, VE response to training was not different between the control and the experimental group since no significant interaction between time, intensity, and intervention was observed for T18 and T38.

MAP was reduced ($P < 0.05$) in the T38 compared with T18 trials (Fig. 5*B*). In the T38 trial, after a significant time*intervention interaction, post hoc analysis revealed a significantly increased ($P < 0.05$) MAP after training for the experimental but not for the control group ($P = 0.98$). For the T18 trial, no such differences between the experimental and the control group could be demonstrated.

$\Delta MCAv_{mean}$ (%baseline) for both groups are displayed in Fig. 5*C*. When compared with the control group, in the experimental group percent baseline $MCAv_{mean}$ was significantly enhanced ($P < 0.05$) after heat training in the T38 trial but not in the T18 trial. In absolute terms, resting and maximal $MCAv_{mean}$ was reduced ($P < 0.05$) from 55.5 ± 3.0 to 42.3 ± 2.9 and from 69.6 ± 5.7 to 52.5 ± 3.4 cm/s, respectively, in T38 compared with T18. In the experimental group, exercise training increased ($P < 0.05$) $MCAv_{mean}$ in T38 and reached similar absolute resting and maximal values as in the T18 trial.

In line with a reduced $MCAv_{mean}$, cerebral oxygenation was also decreased ($P < 0.05$) in the T38 compared with the T18 trials in both the experimental and the control group (Fig. 5*D*). However, the enhanced $MCAv_{mean}$ after heat training did not improve cerebral oxygenation, and also in the control group cerebral oxygenation remained unchanged in the T18 and the T38 trial since neither the main effects for time and intervention nor any interaction was significant.

Cerebrovascular conductance (CVC; $MCAv_{mean}$/MAP) demonstrated no significant change from pre- to postheat acclimation in the T38 trial ($P = 0.08$ for time, $P = 0.27$ for intervention, $P = 0.85$ for time*intervention). In the T18 trial a main effect of time could be observed ($P < 0.05$); however, no significant interaction between time and intervention was demonstrated ($P = 0.26$).

Sweat output and electrolyte concentration during training. Sweat output and sweat electrolyte concentration on *Training Days 1* and *10* in both environmental conditions are presented in Table 3. In the experimental group [Na^+] decreased by 19 ± 7% ($P < 0.05$, $d > 0.80$) from *day 1* to *day 10*, whereas no other parameter was changed as a result of either training intervention.

Sweat output ANOVA results revealed a significant main effect of time and intervention as well as a significant interaction of these two. Mean sweat output in the experimental group was higher ($P < 0.05$, $d > 0.80$) compared with that of the control group during trainings on *days 1* and *10*. Furthermore, a 26 ± 6% increase ($P < 0.05$, $d > 0.80$) in sweat output from *day 1* to *10* was found in the experimental group. In contrast, in the control group an unaltered ($P = 0.43$, $d = 0.12$) water loss was observed from *day 1* to *day 10*.

Correlations. Training-induced relative percent changes in PV, sweat output and electrolyte concentration, $MCAv_{mean}$, HR, $\dot{Q}$ and T_{rec} were analyzed for correlation with the concomitant occurring gain in exercise performance. However, no correlation reached significance (*P* value ranging from 0.18 for PV to 0.82 for sweat output) and Pearson correlation coefficient ranging from 0.1 for $\dot{Q}_{max}$ to 0.3 for PV, which can be considered as small to moderate correlations.

DISCUSSION

The main findings in the present study are *1*) heat training facilitated $\dot{V}O_{2max}$ and Time Trial exercise performance in the heat (38°C) but not in normal (18°C) thermal conditions; *2*) improved exercise performance did not correlate with adaptations in $MCAv_{mean}$ (i.e., cerebral perfusion) as well as PV, sweat output, and sweat [Na^+] following heat training; and finally *3*) acute expansion of PV with albumin infusion did not facilitate exercise performance in 38°C.

Level of heat acclimation. Heat acclimation largely depends on the magnitude of heat stress, duration of exposures, frequency, and total number of stimuli, but it is also well-accepted that most adaptations to heat stress are completed after 7–10 days of daily exposure (46). Typical indicators that sufficient heat acclimation has occurred include a reduced HR, T_{core}, and sweat electrolyte concentration but also an increased PV and sweat output. In the present study, we decided to passively preheat participants before the maximal incremental exercise and the Time Trial tests to always have the same initial T_{rec}. This experimental approach allowed us to examine the impact of heat acclimation at standardized heat strain conditions. This approach, furthermore, assured that participants initiated all exercise tests in a hyperthermic condition since $\dot{V}O_{2max}$ might be maintained with heat exposure if initiated with a normothermic body temperature and also Time Trial performance in the heat might remain unaltered for the first minutes (30). As

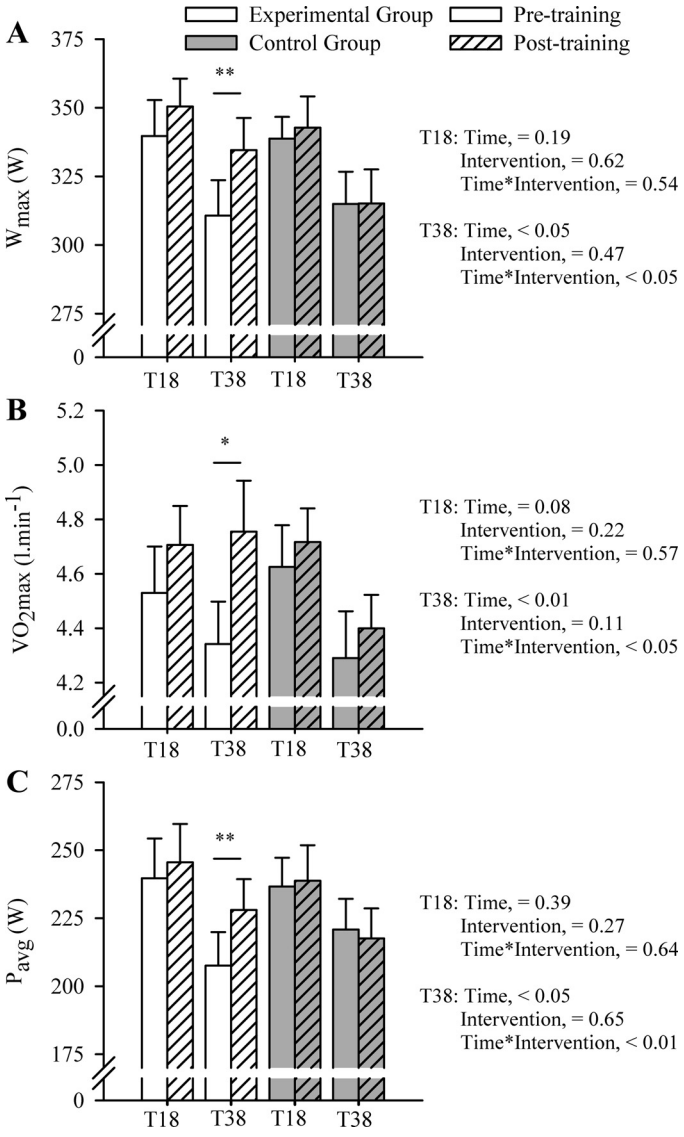

Fig. 4. Absolute pre- and posttraining responses for the experimental and the control group in maximal achieved. W_{max} (in W; *A*), $\dot{V}O_{2max}$ (in l/min; *B*), and P_{avg} (in W; *C*) in temperate conditions (T18) and hot conditions (T38) are shown. Time refers to pre- vs. posttraining, and intervention refers to experimental vs. control group. Values are means ± SE. $*P < 0.05$ and $**P < 0.01$, pre- vs. posttraining within environmental condition; $N = 7$ for the experimental group and $N = 8$ for the control group.

will be discussed later, we also applied this approach to have a similar study design to that of Lorenzo and coworkers (21). In contrast, choosing such study design will most likely have blunted the potential acclimation induced reductions in submaximal T_{rec} and HR. However, because we observed an increase in PV and sweat output and a concomitant reduction in sweat [Na^+] together with an improved exercise performance at 38°C, we are confident that significant heat acclimation has taken place in the study participants.

Exercise performance in 18° and 38°C following heat training. As expected (21, 45), in passively preheated participants, acute heat exposure led to a 4.1 ± 1.4% and 13.1 ± 2.9% reduction in $\dot{V}O_{2max}$ and Time Trial performance, respectively. Also, the observed increase in $\dot{V}O_{2max}$ and Time Trial performance corresponding to 9.6 ± 2.1% and 10.4 ± 3.1% following heat training concurs with previous findings (21, 22). Of note is that the increase in $\dot{V}O_{2max}$ fully compensated the initial heat-induced decrement, and also W_{max} and P_{avg} after heat training were almost completely restored to levels initially obtained at 18°C. So far this has only been observed in the field (34). Collectively, the accumulated evidence emphasizes the need for heat acclimation to maximize performance in a hot environment and, furthermore, suggests that acclimation can at least be acquired partially without traveling to the site of competition. However, heat training in the present study did not facilitate exercise performance when tested in 18°C ambient conditions. In a recent review Corbett et al. (6) analyzed several studies investigating the effects of acclimation on exercise performance in temperate and cold conditions. Although some studies have reported an ergogenic effect of acclimation, most of the findings have been confounded by several factors such as the absence of a control group, the inclusion of untrained subjects, suboptimal acclimation programs, or the application of an unclear study design. The most convincing study mentioned in this review was conducted by Lorenzo et al. (21). Although we applied a similar study design, they found heat training-induced gains of 5% and 6% for $\dot{V}O_{2max}$ and Time Trial performance, respectively, when tested at 13°C (21), which contrasts our study. In this regard, it seems unlikely that such divergence in performance is related to the temperature difference (18°C vs. 13°C) in which the participants were tested (16). In addition, the degree of adaptations to heat exposure has previously been linked to training status (41). Nonetheless, the participants in the latter study were well-trained ($\dot{V}O_{2max} \approx 67$ ml·min^{-1}·kg^{-1}) and hence of similar fitness levels to the volunteers included in the present study ($\dot{V}O_{2max} \approx 61$ ml·min^{-1}·kg^{-1}). Otherwise, it is noteworthy that in the aforementioned study (21) the training sessions in the heat were conducted at a relatively higher exercise intensity when compared with the training conducted in the control trials. In the present study all trainings were matched to elicit the same relative cardiovascular strain. Although the difference was rather small (~20%), this may explain, at least in part, the difference in study outcomes. The finding that exercise performance following heat acclimatization is not enhanced in a temperate environment is in agreement with a controlled study performed in competitive cyclists ($\dot{V}O_{2max} \approx 63$ ml·min^{-1}·kg^{-1}) (16). Here it was demonstrated that when compared with control training, no additional benefit of 2 wk of heat training was apparent when tested in 5–13°C, whereas an expected effect was observed when the exercise tests were conducted at 35°C.

Lorenzo and coworkers (21) speculated that the observed increase in PV (estimated from changes in Htc) of ≈200 ml could have facilitated exercise capacity in 13°C. Herein, the heat training-induced increase in PV of 201 ± 88 ml did not facilitate exercise performance in 18°C, which adds to the controversy on whether PV expansion facilitates $\dot{V}O_{2max}$ or Time Trial performance in temperate conditions (7, 47). Although there is little doubt that PV expansion can enhance $\dot{Q}_{max}$ (3), the concomitant-induced hemodilution has to be counterbalanced by an even greater increase in $\dot{Q}_{max}$ to increase exercise performance. In the present study, the increase in PV of 15.7 ± 0.6% led to an 18.2 ± 2.1% increase in $\dot{Q}_{max}$ and a concomitant 8.2 ± 0.1% reduction in

Table 2. *Cardiovascular and thermoregulatory responses to pre- and posttraining $\dot{V}O_{2max}$ and 30-min time trial tests in temperate conditions (T18) and hot conditions (T38) in the EG and CG*

	$\dot{V}O_{2max}$ Test				Time Trial Test						
	EG		CG		EG		CG		*P* Value		
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Time	Intervention	Interaction
$\dot{Q}_{80W}$, l/min											
18°C	14.0 ± 0.9	13.3 ± 0.7	14.0 ± 0.4	13.3 ± 0.6	—	—	—	—	0.29/−	0.39/−	0.72/−
38°C	15.3 ± 0.6	15.3 ± 0.6	14.2 ± 0.6	15.1 ± 0.7	—	—	—	—	0.67/−	0.06/−	0.88/−
$\dot{Q}_{peak}$, l/min											
18°C	20.6 ± 0.8	20.3 ± 1.0	20.8 ± 0.7	19.9 ± 0.9	—	—	—	—	0.15/−	0.77/−	0.57/−
38°C	21.3 ± 0.6	21.7 ± 0.5	20.0 ± 1.1	20.1 ± 0.8	—	—	—	—	0.29/−	0.25/−	0.22/−
SV_{80W}, ml											
18°C	146 ± 8	139 ± 8	133 ± 6	135 ± 7	—	—	—	—	0.62/−	<0.05/−	0.60/−
38°C	135 ± 5	138 ± 9	127 ± 9	130 ± 6	—	—	—	—	0.87/−	0.05/−	0.81/−
SV_{peak}, ml											
18°C	131 ± 5	129 ± 5	122 ± 5	121 ± 5	—	—	—	—	0.79/−	0.06/−	0.08/−
38°C	129 ± 7	131 ± 6	118 ± 7	117 ± 5	—	—	—	—	0.63/−	0.34/−	0.96/−
Resting T_{rec}, °C											
18°C	38.0 ± 0.0	37.8 ± 0.0	37.9 ± 0.2	37.7 ± 0.2	37.9 ± 0.0	37.7 ± 0.1	37.9 ± 0.1	37.6 ± 0.1	<0.05/<0.05	0.31/0.29	0.97/0.49
38°C	38.7 ± 0.1	38.6 ± 0.1	38.6 ± 0.1	38.7 ± 0.1	38.5 ± 0.2	38.7 ± 0.1	38.6 ± 0.2	38.7 ± 0.0	0.94/0.22	0.63/0.63	0.19/0.98
Maximal T_{rec}, °C											
18°C	38.7 ± 0.1	38.5 ± 0.1	38.4 ± 0.2	38.4 ± 0.1	39.2 ± 0.1	38.8 ± 0.2	39.2 ± 0.1	38.6 ± 0.3	0.57/<0.05	0.27/0.31	0.45/0.34
38°C	38.8 ± 0.1	38.8 ± 0.1	38.7 ± 0.1	38.7 ± 0.1	39.7 ± 0.2	39.6 ± 0.2	39.5 ± 0.1	39.6 ± 0.1	0.53/0.71	0.31/0.59	0.86/0.51
Resting T_{skin}, °C											
18°C	34.2 ± 0.2	32.5 ± 0.7	34.0 ± 0.2	33.0 ± 0.6	33.3 ± 0.6	32.6 ± 0.4	32.6 ± 0.4	32.7 ± 0.7	0.06/0.53	0.50/0.52	0.26/0.46
38°C	36.5 ± 0.5	36.4 ± 0.4	36.2 ± 0.3	36.7 ± 0.4	36.0 ± 0.6	35.9 ± 0.4	36.0 ± 0.6	36.6 ± 0.4	0.59/0.30	0.97/0.71	0.46/0.68
Maximal T_{skin}, °C											
18°C	35.5 ± 0.6	34.9 ± 1.0	35.9 ± 0.4	34.6 ± 1.1	36.2 ± 0.6	35.6 ± 0.8	36.2 ± 0.3	35.4 ± 1.1	0.25/0.47	0.92/0.80	0.69/0.86
38°C	37.2 ± 0.3	36.8 ± 0.3	37.5 ± 0.3	37.9 ± 0.3	37.7 ± 0.5	37.3 ± 0.1	37.8 ± 0.4	38.0 ± 0.2	0.99/0.75	<0.05/0.47	0.19/0.61
Resting HR, beats/min											
18°C	68 ± 4	65 ± 4	66 ± 5	65 ± 6	73 ± 10	67 ± 4	68 ± 5	70 ± 6	0.36/0.52	0.64/0.96	0.87/0.45
38°C	75 ± 3	75 ± 4	70 ± 4	73 ± 4	79 ± 3	80 ± 3	79 ± 7	83 ± 4	0.51/0.91	0.29/0.40	0.70/0.60
Maximal HR, beats/min											
18°C	180 ± 2	176 ± 2	177 ± 3	178 ± 1	182 ± 4	177 ± 3	180 ± 1	180 ± 2	0.40/0.20	0.82/0.84	0.25/0.16
38°C	179 ± 2	181 ± 2	180 ± 2	180 ± 1	183 ± 4	186 ± 2	181 ± 2	179 ± 4	0.32/0.96	0.99/0.05	0.60/0.23

Values are means ± SE; $N = 7$ for the experimental group (EG) and $N = 8$ for the control group (CG). $\dot{Q}_{80W}$, cardiac output at 80 W; $\dot{Q}_{max}$, maximal cardiac output; SV_{80W}, stroke volume at 80 W; SV_{max}, maximal stroke volume; HR, heart rate; Pre, pretraining; post, posttraining. *P* values for $\dot{V}O_{2max}$ test/time trial test are shown.

Hct. Furthermore, as will be discussed below, the expansion of PV did not enhance exercise performance in a hot environment (48).

Therefore, if an appropriate study design and controlling for training load is applied, we suggest that heat training does not facilitate exercise performance in a temperate environment any more than ordinary exercise training does.

Heat training-induced increase in plasma volume and exercise performance in the heat. The first speculations that BV might increase in response to a rise in ambient temperature is believed to date back to 1923 (2). Since then, numerous studies have confirmed that exposure to warm environments increases PV after a few days of acclimation, and even so if the length of heat exposure is as little as 1 h/day (23, 24, 40, 49). Hypervolemia is often suggested to be the most important feature of heat acclimation (24, 40). In the current study heat training augmented PV by 6 ± 2%, which is in agreement with previous findings (21, 23). PV was not increased in the control group. In previously untrained individuals, PV usually increases within a few days of exercise training regardless of the thermal environment (1, 14). The reason for the control training not to expand PV is likely related to the already relative fit status of the included participants. Moreover, in the current study, the heat training-induced increase in resting PV was not statistically associated with the concomitantly occurring improvements in W_{max}, $\dot{V}O_{2max}$, or Time Trial performance, which is in line with previous findings (32, 33). However, these studies also reported significant correlations between acclimatization-induced changes in exercise performance and dynamic changes in PV while exercising in the heat, suggesting that PV retention during exercise is more important than absolute resting PV. Of note is that whereas $\dot{V}O_{2max}$ when tested in 38°C was increased by 9% following heat training, $\dot{Q}_{max}$ remained nearly unchanged (21.3 to 21.7 l/min). Because maximal O_2 extraction across the exercising skeletal muscle is reported unchanged following heat training (23, 24), we assume that the ≈400 ml numerical higher $\dot{Q}_{max}$ may at least be partially responsible for the increase in $\dot{V}O_{2max}$, but that statistical power may have lacked to establish this association. We acknowledge that 400 ml of extra $\dot{Q}_{max}$ cannot explain the entire 400 ml elevation in $\dot{V}O_{2max}$; we cannot, however, account for the remaining part. The modest increase in $\dot{Q}_{max}$ was likely the result of the also modest increase in PV (≈200 ml), whereas in a previous study the removal of 382 ml of whole blood reduced

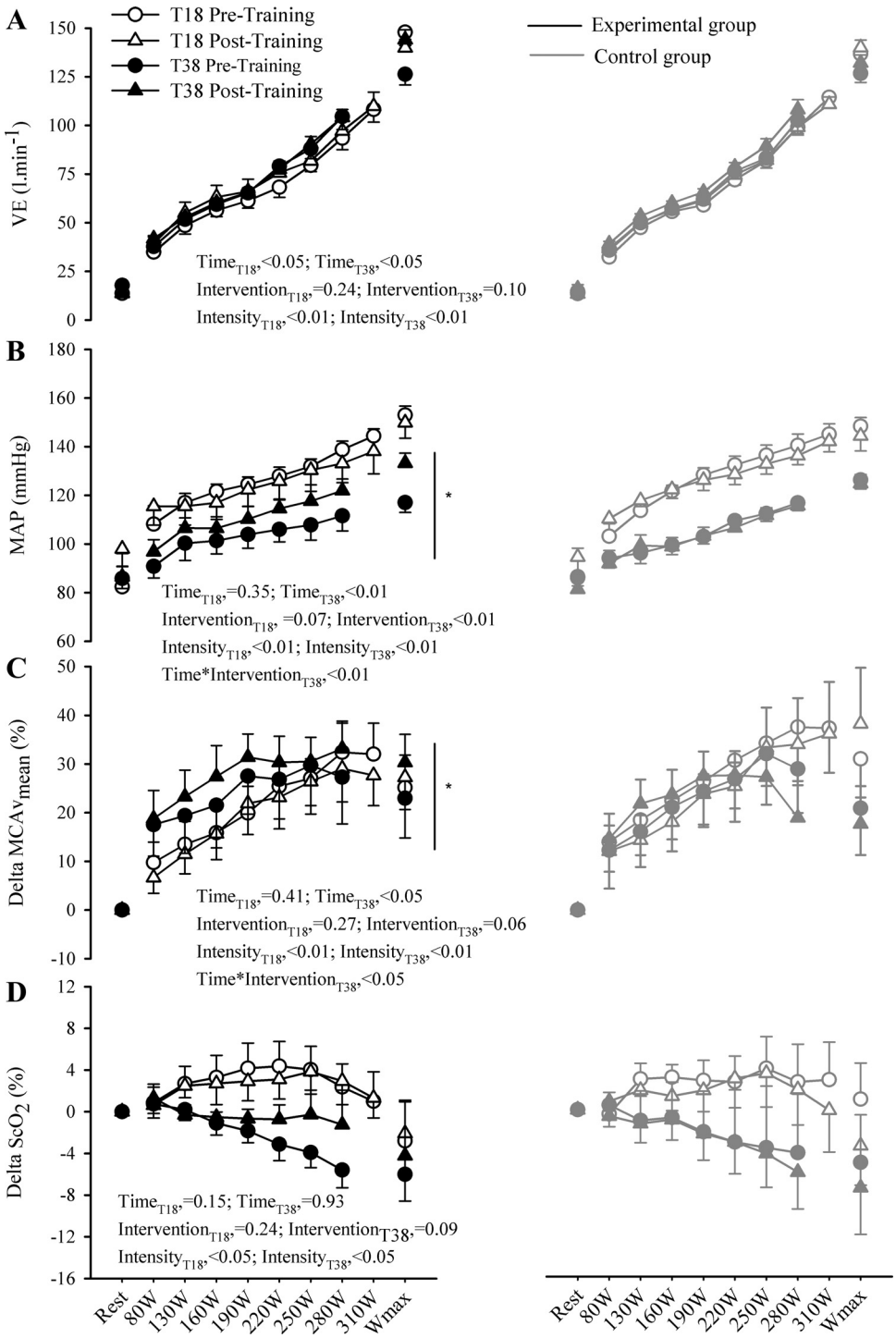

Fig. 5. Ventilation (VE; in l/min) (*A*), mean arterial pressure (MAP; in mmHg) (*B*), changes in middle cerebral artery velocity ($\Delta MCAv_{mean}$; in percentage) (*C*), and changes in cerebral oxygenation (ΔSco_2; in percentage) (*D*) in temperate conditions (T18) and hot conditions (T38) for the experimental group (black lines) and the control group (gray lines). Values are means ± SE. $*P < 0.05$, overall pre- vs. posttraining intervention for T38; $N = 7$ for the experimental group and $N = 8$ for the control group for *A*–*C*. For *D*, $N = 4$ (experimental and control group) for the T38 trial and $N = 6$ (experimental group) and $N = 7$ (control group) for the T18 trial.

$\dot{Q}_{max}$ by 2.3 l/min (3). In the present study, the acute expansion of PV by 538 ± 16 ml (15%) of albumin solution did not facilitate exercise performance with acute heat exposure, which is in agreement with the only previous study that applied a similar experimental approach (48). Nevertheless, in the present study PV expansion by albumin led to an increase in submaximal and maximal SV and $\dot{Q}$. We speculate that $\dot{V}O_{2max}$ and Time Trial performance were not enhanced despite these improvements, since PV expansion also prompted an 8% reduction of Htc, and thereby likely offset some of the potential blood flow-dependent improvements in O_2 transport to the exercising skeletal muscles. An interesting point would be to see whether artificial PV expansion might have a different influence on highly dehydrated subjects, where hemoconcentration has occurred. Indication, therefore, is given by the fact that fluid ingestion during exercise in the heat decelerates fatigue. Dehydration-induced reduction in skin and locomotor muscle blood flow rapidly lowers O_2 delivery to the exercising legs and the capacity of heat dissipation (11). Therefore, a higher PV might have a beneficial effect on exercise capacity

Table 3. *Sweat composition and sweat rate collected during the first training day (Day 1) and the last training day (Day 10) in the experimental and the control groups*

	Experimental Group		Control Group		*P* Value		
	Day 1	*Day 10*	*Day 1*	*Day 10*	Time	Intervention	Interaction
Mean concentration, mmol/l							
Sodium	167 ± 22	127 ± 10*	119 ± 16	123 ± 14	<0.05	0.16	<0.05
Calcium	0.7 ± 0.2	0.9 ± 0.2	1.1 ± 0.3	0.9 ± 0.1	0.85	0.39	0.31
Potassium	8.1 ± 2.2	8.3 ± 2.3	12.8 ± 3.0	11.2 ± 1.9	0.80	0.13	0.60
Chloride	159 ± 23	136 ± 20	112 ± 13	113 ± 14	0.60	0.10	0.55
Sweat rate, kg/h	1.44 ± 0.10	1.74 ± 0.11*	0.82 ± 0.09†	0.83 ± 0.23†	<0.05	<0.01	<0.05

Values are means ± SE; $N = 7$ for the experimental group and $N = 8$ for the control group. *$P < 0.05$ vs. *Day 1*; †$P < 0.05$ vs. experimental group.

by increasing blood flow to the important organs without lowering Hct. However, this remains to be established.

Acclimation-induced normalization of cerebral perfusion (i.e., $MCAv_{mean}$) and its relation to exercise performance. As expected (26), acute exposure to 38°C reduced $MCAv_{mean}$ during exercise, whereas heat training facilitated $MCAv_{mean}$ during exercise at 38°C to such an extent that it became restored to levels observed with exercise at 18°C. This has not been demonstrated previously. $MCAv_{mean}$ is regulated by various factors, where $PaCO_2$ (ventilation) and MAP are the most important ones (31). VE was not reduced with heat training and was hence not associated with the reduction in $MCAv_{mean}$ ($R = 0.23$), which is in line with previous findings (10). On the other hand we found MAP to be increased with heat training. With exercise in the heat, blood flow to the skin is facilitated to favor cooling (12, 36), and this will inevitably lead to a reduction in blood availability for other organs such as the brain (26). It could hence be speculated that the increase in plasma volume observed with heat training could lead to increased MAP, restored cerebral perfusion, and preserved skin perfusion, although this depends on the interplay between the degree of vasodilation and the volume increase in plasma. In the present study, the association between increases in PV and $MCAv_{mean}$ and PV and MAP was $R = 0.26$ and $R = 0.20$ ($P = 0.57$ and $P = 0.43$), respectively, with a tendency for a moderate association between $MCAv_{mean}$ and MAP ($R = 0.41$; $P = 0.07$). Therefore, the mechanisms facilitating $MCAv_{mean}$ with heat acclimation could not be determined. Nonetheless, it should be noted that CVC was not different between pre- and postheat acclimation measurements. Hence, when MAP was accounted for, heat acclimation does not alter $MCAv_{mean}$, which suggests that changes in $MCAv_{mean}$ can be mainly be attributed to changes in MAP.

An augmented $MCAv_{mean}$ could facilitate exercise capacity by several mechanisms. Cerebral hypoxemia has been suggested to facilitate centrally mediated fatigue (35), and a restoration of cerebral oxygenation secondary to enhancing brain blood flow has hence been proposed as a candidate to facilitate exercise performance. However, although the proposed mechanism has received much attention within the last decade (26, 27, 35), in studies in which $MCAv_{mean}$ and cerebral oxygenation have been increased by the administration of small volumes of CO_2 to the inspiration in hypoxia, this has not improved exercise performance (8, 43), also with additional heat exposure (17). It has to be mentioned that when cerebral blood flow (e.g., with CO_2) was artificially manipulated, simultaneously other factors such as pH, ventilation, and breathing resistance will be affected, which all could negatively influence exercise performance. Alternatively, an improved cerebral perfusion has been suggested to favor cooling of the brain (28) and thereby improve exercise capacity in the heat, but this cannot be addressed in the current study.

As mentioned, MAP was reduced at 38°C in the study participants. MAP is generally reported being maintained with heat exposure until dehydration occurs (13). Although we cannot exclude that dehydration may have occurred, this seems unlikely since the duration of the $\dot{V}O_{2max}$ test was less than 20 min. One possible explanation for the reduced MAP could be related to our preheating protocol where participants intentionally initiated the exercise tests hyperthermic, which in turn could have decreased MAP. However, because this observation was similarly present before and after the training and in the experimental and the control group, it should not have influenced our finding that MAP was increased by heat training.

Heat training facilitated sweat rate and lowered sweat electrolyte concentration and their effect on exercise performance in the heat. With heat training, a substantial increase in sweat output (+26 ± 6%) and decrease in sweat [Na+] (−19 ± 7%) occurred. This is in line with previous research (16, 24) and indicates that substantial heat acclimation had occurred. Despite that these have previously been suggested to facilitate exercise performance in the heat, no such correlations could be established in the current study. It could be speculated, however, that at least the more diluted sweat could become advantageous especially for longer lasting submaximal events. It also needs to be mentioned that the increased sweat output likely minimally increased evaporative heat loss, since sweat rate was certainly beyond the maximal evaporative capacity of a 38°C environment (i.e., low sweating efficiency).

Limitations to the study. Due to the limited sample size, it cannot be ruled out that a statistical type II error prevented the correlational analysis to reveal associations between the observed performance gains and the measured physiological variables, which also applies to the ANOVA testing. As already mentioned, because we raised the participants' T_{rec} to always the same initial value, part of the potential adaptations, such as submaximal T_{core} and HR, may have become blunted. Furthermore, a possible acclimation decay has to be considered. Although the T38 trial was deliberately conducted before the T18 trial to have an additional heat stimulus during the 4- to 5-day posttesting period, it cannot be excluded that partial de-acclimation in the T18 trial has occurred and, therefore, influenced our results. Other limitations to the study are that the included study volunteers were not blinded toward the treatment. It is, however, virtually impossible to blind humans when it comes to heat exposure, and the use of a cross-over

study design was judged the best solution. It would have been advantageous to blind the investigators toward the treatment also, but due to staff limitations this was, unfortunately, impossible. Furthermore, it has to be mentioned that the artificial PV increase was higher (15%) than the actual heat training-induced increase (6%). The aim was to expand PV equal to or more than what normally occurs with heat training since if we were to expand by too little a negative result could be argued to derive from too little of an expansion. Some of our study participants displayed an increase in PV of up to 13.5%, which further strengthens this assumption. However, we acknowledge that the higher PV infusion might have led to such hemodilution, which might have offset a potential beneficial effect of an elevated $\dot{Q}$. Besides, it needs to be acknowledged that $MCAv_{mean}$ is only a surrogate measure of cerebral perfusion and that hyperthermia-induced vasconstriction may have influenced those results. Also the recording of cerebral oxygenation (NIRS) in the heat proves to be challenging and has to be interpreted with caution. Interfering sweat can cause missing values, and also skin blood flow may have introduced bias since this greatly influences cerebral NIRS signal (44). Furthermore, it needs to be acknowledged that also the measurement of $\dot{Q}_{max}$ with the Innocor device has its limitations since it highly depends on the participants' ability to follow the given breathing pattern. Recently, however, we demonstrated the devices ability for repeated measures (42). Finally, as already mentioned, it has to be acknowledged that in some participants not allowing drinking might have led to hypohydration even though exercise tests were always initiated well-hydrated.

The novel findings of the present study are that 10 days of heat training facilitated exercise performance in the heat but not in temperate conditions. Furthermore, adaptations in PV, sweat output, and sweat $[Na^+]$ and cerebral perfusion, which have previously been proposed to facilitate exercise performance in the heat, were not associated with the observed gains in performance. Finally, in well-trained subjects acute expansion of plasma volume with albumin infusion did not facilitate exercise performance in 38°C.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

S.K. and C.L. conception and design of research; S.K., D.F., F.H., A.S., and M.P.H. performed experiments; S.K. analyzed data; S.K. and C.L. interpreted results of experiments; S.K. prepared figures; S.K. and C.L. drafted manuscript; S.K., D.F., F.H., A.S., M.P.H., and C.L. approved final version of manuscript; D.F., F.H., A.S., and M.P.H. edited and revised manuscript.

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ORIGINAL ARTICLE

The carbon monoxide re-breathing method can underestimate Hb_{mass} due to incomplete blood mixing

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Abstract
Purpose Hemoglobin mass (Hb_{mass}) is commonly assessed using the CO re-breathing method with the subject in the seated position. This may lead to an underestimation of Hb_{mass} as blood in lower extremity veins while seated may not be tagged with carbon monoxide (CO) during the re-breathing period.
Methods To test this hypothesis, CO re-breathing was performed on four occasions in nine male subjects, twice in the seated position and twice in combination with light cycle ergometer exercise (1 W/kg body-weight) intending to accelerate blood circulation and thereby potentially allowing for a better distribution of CO throughout the circulation as compared to in the seated position. Blood samples were drawn from an antecubital vein and the saphenous magna vein following the re-breathing procedure.
Results In the seated position, CO re-breathing increased the percent carboxyhemoglobin (%HbCO) in the antecubital vein to 8.9 % (7.8–10.7) [median (min–max)], but less ($P = 0.017$) in the saphenous magna vein [7.8 % (5.0–9.9)]. With exercise, no differences in %HbCO were observed between sampling sites. As a result, CO re-breathing in combination with exercise revealed a ~3 % higher ($P = 0.008$) Hb_{mass}, i.e., 936 g (757–1,018) as compared to 908 g (718–940) at seated rest.
Conclusion This study suggests an uneven distribution of CO in the circulation if the CO re-breathing procedure is performed at rest in the seated position and therefore can underestimate Hb_{mass}.

Keywords nHb · Red cell mass · Blood volume · CO · RCV

Introduction

Methods that quantify hemoglobin mass (Hb_{mass}) or red blood cell volume (RCV) in humans are based on the dilution principle. A given quantity of tracer is administered to the circulation, and the subsequent concentration in the blood allows for calculation of Hb_{mass} or RCV. The use of radioactive tracers such as ^{51}Cr is considered the gold standard (International Committee for Standardization in Haematology 1980). However, also carbon monoxide (CO) is commonly used because of its ease of use. The CO re-breathing method is associated with a typical measurement error similar to the ^{51}Cr method (Gore et al. 2005) and is considered as valid and reliable (Ashenden et al. 1999; Burge and Skinner 1995; Hutler et al. 2000; Thomsen et al. 1991). A prerequisite for any dilution principle is a uniform distribution of the tracer, i.e., the pulmonary administered CO needs to be distributed evenly throughout the circulation. Since there is no transfer of CO between hemoglobin molecules (Blackmore 1970), only red blood cells passing

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the pulmonary circulation may be tagged with CO. The CO re-breathing procedure is commonly conducted at seated rest (Lundby et al. 2007; Morkeberg et al. 2011; Prommer et al. 2008; Robach et al. 2006; Schmidt and Prommer 2005; Steiner and Wehrlin 2011; Ulrich et al. 2011) or semi-recumbent rest (Garvican et al. 2010). In the supine position, total blood turnover in large veins may take up to 30 min (Wennesland et al. 1962), and likely even longer in the seated position as gravitation promotes blood accumulation in the leg veins. If erythrocytes remain in lower extremities during a CO re-breathing procedure, they cannot be tagged with CO which may lead to an underestimation of Hb_{mass}. Complete blood mixing has been suggested to occur within 6–10 min based on similar percent carboxyhemoglobin (%HbCO) values in blood samples obtained from the radial artery, earlobe capillaries and an antecubital vein (Garvican et al. 2010; Prommer and Schmidt 2007). These studies, however, did not include blood sampling from large veins of the lower body.

We speculated that a non-uniform distribution of CO may be prevented if the CO re-breathing procedure is performed in combination with light exercise. Exercise mobilizes venous blood through the muscle pump effect. This may facilitate blood pooled in the lower extremities to pass the pulmonary circulation and accordingly be tagged with CO.

We thus hypothesized that (i) CO re-breathing performed in the resting seated position will lead to higher %HbCO values in an antecubital vein than in the saphena magna vein of the lower leg, (ii) exercise abolishes this difference by enhancing blood circulation and, as a consequence, (iii) the implementation of light exercise to the CO re-breathing procedure reveals a higher Hb_{mass} suggesting that CO re-breathing performed in the resting seated position underestimates Hb_{mass}.

Methods

This study was approved by the Research Ethics Committee of ETH Zurich, Switzerland (EK 2012-N-07) and the experimental protocols conformed to the Declaration of Helsinki. All subjects gave oral and written informed consent before participation. Subjects were excluded from the study if they had donated blood within the last 3 months prior to the study and were not allowed to donate blood before the study was completed.

Subjects

Nine healthy men (26.7 ± 3.8 years, 1.81 ± 0.08 m, 75.6 ± 6.0 kg, mean ± SD) were included as subjects. All subjects were recreationally physically active but were not involved in exercise training at a high level.

Study design

Subjects reported to the laboratory on four consecutive days. They performed two CO re-breathing maneuvers at rest in the seated position and two in combination with mild exercise. To exclude a cumulative effect of administered CO, all measurements were separated by an interval of at least 24 h, which is sufficient as judged by the pre %HbCO values.

Protocol

The first measurement was always carried out at rest to allow for familiarization without the stress of exercise. The order of the following three measurements was randomized.

Resting seated CO re-breathing

The seated CO re-breathing was performed in a slightly modified version of the Burge and Skinner (1995) protocol. The re-breathing system was checked for leaks by water submersion prior to use. A 20-G catheter was placed in an antecubital and in the saphena magna vein at the ankle when possible, alternatively, a butterfly needle was used. The distance from the sampling side to the heart was about 30–40 cm and 90–110 cm, respectively. After placement of the catheter, the stasis was removed and care was taken not to apply any pressure on the subject to maintain venous blood flow. The subjects then rested in the seated position for 20 min with the arms hanging loosely down. During this time, they ingested 500 ml of water to stabilize plasma volume. The subjects thereafter breathed 100 % O_2 for 4 min through an open circuit system (100 l Douglas bag) to eliminate nitrogen from the airways. Thereafter, a 2-ml blood sample was drawn from the antecubital vein and analyzed in quadruplicate for %HbCO and Hb concentration ([Hb]) on a hemoximeter (ABL800, Radiometer, Copenhagen, Denmark) and for hematocrit (Hct) by the micro-method (4 min at 13,500 rpm). The open circuit was then switched to a closed re-breathing circuit (previously flushed and filled with O_2) with an integrated CO_2 absorber containing 1 kg of soda lime and 4 l re-breathing bag. After a few breaths, a 200-ml syringe with 1.5 ml/kg body mass of 99.997 % chemically pure CO (CO N47, Air Liquide, Pullach, Germany) was administered to the circuit and re-breathed for 10 min. At the end of the re-breathing period, a second blood sample was obtained from the antecubital vein. Simultaneously, a further blood sample was obtained from the saphena magna vein (only after the first resting seated and exercise protocols, respectively, whereas the saphena magna vein was not punctured in the other two measurements). Both samples were analyzed as the initial blood sample.

Exercise CO re-breathing

The CO re-breathing procedures performed during exercise was carried out as in the seated position for all main points. After completing the same 20 min of rest and the consumption of 500 ml of water, the subjects were moved from the chair to a cycle ergometer (Monark Ergomedic 839 E, Sweden). The 4 min of 100 % O_2 breathing was initiated simultaneously with the onset of light exercise (1 W/kg body-weight). The subjects continued to exercise throughout the entire re-breathing protocol and blood sampling. At the end of the 4 min period, a blood sample was obtained from the antecubital vein and subjects were switched to the closed circuit. After a short familiarization period, an identical amount of CO as in the resting seated protocol was administered and re-breathed for 10 min. At the end of the re-breathing period, blood samples were drawn simultaneously from the antecubital and from the saphena magna vein (only after the first exercise measurement). All CO re-breathing procedures were performed by the same operator.

Additional validations studies

Since CO clearance from the circulation is increased with hours of elevated pulmonary ventilation (Bruce and Bruce 2006), we tested the potential bias (increased Hb_{mass}) introduced to the measurement by the increased VE during exercise. It is important to realize, however, that our measurements are completed with the subjects connected to a closed system, and hence any CO potentially cleared by a higher ventilation will be re-breathed and thus the likelihood for a bias seems minor. The proof-of-concept validation was done in four separate subjects not taking part in other parts of the experiments. The above-described CO re-breathing procedure was performed on two separate days in these subjects (1) with normal voluntarily VE and (2) with subjects hyperventilating at $\approx$30 l/min (measured with a Quark b2, Cosmed, Italy). %HbCO values in the blood samples obtained from the antecubital vein after 10 min of CO re-breathing were as follows: subject 1: 7.3 vs 7.2 %; subject 2: 8.2 vs 8.4 %; subject 3: 9.7 vs 9.9 %; and subject 4: 8.1 vs 7.9 % for measurements completed on Day 1 (normal VE) vs Day 2 (hyperventilation), respectively. Hence the increased VE during exercise does not seem to affect our measurements.

Calculations

The mean difference in %HbCO between the first and the second blood samples was used to calculate Hb_{mass}. RCV, plasma volume (PV) and blood volume (BV) were then derived using Hb_{mass}, [Hb] and Hct. The blood variables were calculated using standard equations (Burge and Skinner 1995).

Values from day one from the seated and the exercise protocol were used to calculate the difference in %HbCO between the antecubital and the saphena magna vein. The second trial of both methods was used for TE and Hb_{mass} calculations. The latter values represent the mean value from the 2 days of each protocol.

Statistics

Statistical evaluations of the data were performed using the software IBM SPSS 20. Systematic errors could not be found. As the data were not normally distributed, non-parametric Kolmogorov–Smirnov tests were used to compare the medians. A P value <0.05 was considered statistically significant. The coefficient of variation for the Hb_{mass} for the two testing models was expressed as the typical error, which is calculated as the standard deviation of difference scores divided by $\sqrt{2}$ (Hopkins 2000). When expressed as a percent of the mean, the percent typical error (%TE) is obtained. The results are presented as median (min–max).

Results

Antecubital vs saphena magna vein %HbCO at rest and following exercise

Values for %HbCO are shown in Table 1. It was not possible to puncture the saphena magna vein in one subject. Hence, for this analysis eight subjects were included. The median difference in %HbCO between the antecubital and saphena magna vein sample was 0.55 % (0.20–5.68) in the resting seated position, with the value being higher in the antecubital samples in all subjects (Fig. 1a). With exercise, the difference in %HbCO was 0.01 % (−0.30–0.20) ($P = 0.017$ vs resting protocol).

The effect of exercise (vs rest) CO re-breathing on Hb_{mass} values

Values for Hb_{mass}, [Hb], Hct, and blood volume variables are presented in Table 2. We observed an increase in most blood volume variables when the CO re-breathing was performed during exercise. Figure 1b illustrates the Hb_{mass} yielded by the two protocols. The median Hb_{mass} increased ($P = 0.008$) from 908 g (718–940) measured in the seated position to 936 g (757–1,018) obtained with the exercise protocol. The increase in Hb_{mass} with exercise was a uniform finding across all subjects.

Table 1 Antecubital (AC), saphenous magna (SM) and delta AC SM (ΔAC SM) venous carboxyhemoglobin values (%HbCO) in all subjects after 10 min of CO re-breathing with seated rest (Seated) or light ergometer exercise (Exercise)

Subject	Seated 1			Seated 2		Exercise 1			Exercise 2	
	AC	SM	ΔAC SM	AC	SM	AC	SM	ΔAC SM	AC	SM
1	8.85	7.30	1.55	9.25	–	8.45	8.60	−0.15	8.45	–
2	9.9	9.70	0.20	10.30	–	9.25	9.10	0.15	9.75	–
3	9.55	9.33	0.23	9.45	–	8.65	8.95	−0.30	8.78	–
4	10.70	5.03	5.68	10.98	–	10.00	9.98	0.03	10.08	–
5	9.10	5.03	4.08	8.65	–	8.50	8.40	0.10	8.65	–
6	8.23	8.00	0.23	8.45	–	7.30	7.45	−0.15	7.88	–
7	7.80	7.38	0.43	7.90	–	7.18	7.18	0.00	7.83	–
8	8.90	8.23	0.68	9.10	–	8.60	8.40	0.20	8.33	–
Median	9.00	7.69	0.55*	9.18	–	8.55	8.50	0.01*	8.55	–

* Differences between the measurements ($P < 0.05$)

Reliability of the two methods

The TE was 1.7 % for the resting seated protocol and 2.4 % for the exercise protocol. No statistical difference was found ($P = 0.515$) when comparing the median scores of each method between the first and the second day.

Discussion

This study indicates an uneven distribution of CO in the blood circulation if the CO re-breathing procedure is performed in the resting seated position. In accordance with our hypothesis, we observed higher %HbCO in the antecubital than in the saphenous magna vein following CO re-breathing performed in the resting seated position, whereas this difference was abolished when re-breathing was performed during light cycling exercise.

The increase in saphenous venous %HbCO in the exercise protocol is likely accounted for by a mobilization of blood in the veins of the lower extremities leading to more complete blood mixing. Complete blood mixing has been suggested to occur within 6–10 min based on similar %HbCO values in blood samples obtained from the radial artery, earlobe capillaries and an antecubital vein (Garvican et al. 2010; Prommer and Schmidt 2007). All these studies, however, failed to include blood sampling from large veins of the lower body, and that may explain the difference in results. Our data indicate that complete blood mixing does not occur within a 10-min period.

In addition to enhanced circulation, splenic contraction during exercise might be an additional explanation for the higher Hb_{mass} values obtained during the exercise trial. In humans, ~10 % of Hb_{mass} is stored in the spleen at rest (Stewart and McKenzie 2002). With exercise, a relative decrease in splenic erythrocyte content is a common observation and may reach 40–60 % at maximal workloads (Flamm et al. 1990; Froelich et al. 1988; Laub et al. 1993; Sandler et al. 1984). With light exercise similar to that in the present study, a reduction in splenic blood content of about 10 % has been reported against seated rest (Laub et al. 1993). Assuming a splenic volume of no more than 300 ml, this would result in a 30 ml increase in circulating whole blood. Assuming a splenic Hct of 80 %, a 10 % reduction in splenic blood content will correspond to 25 ml of red cells entering the circulation. Since we observed an increase of 85 ml in RCV in the exercise trial, the contribution of splenic contraction to the observed increase in Hb_{mass} with exercise may account for up to 28 %.

It should be noted that the typical error for the measurements increased (not statistically significant) from 1.7 to 2.4 when using the exercise protocol. Both values are, however, within the expected values for the method (Gore et al. 2005). The source for the increased TE with exercise remains unknown. One limitation of the exercise protocol is the unknown amount of CO that may have remained in the re-breathing circuit after each procedure. We made the assumption (Thomsen et al. 1991) that 2.2 % of the CO remains within the re-breathing circuit and that this is similar for both protocols. However, since exercise accelerates blood circulation and ventilation, the absorbed CO during the 10-min of re-breathing may have been higher during the exercise protocol. However, an underestimation of absorbed CO from the re-breathing circuit would have resulted in an underestimation of Hb_{mass} in the exercise trial and further support our conclusions. Another potential limitation to the exercise (and seated) protocol is the unknown amount of CO that may leave the vascular space and subsequently bind to myoglobin. Based on theoretical reasoning, CO loss from the circulation is estimated to be 1.6 ml CO in 10 min at rest (Garvican et al. 2010). During exercise, more muscle capillaries are opened which may

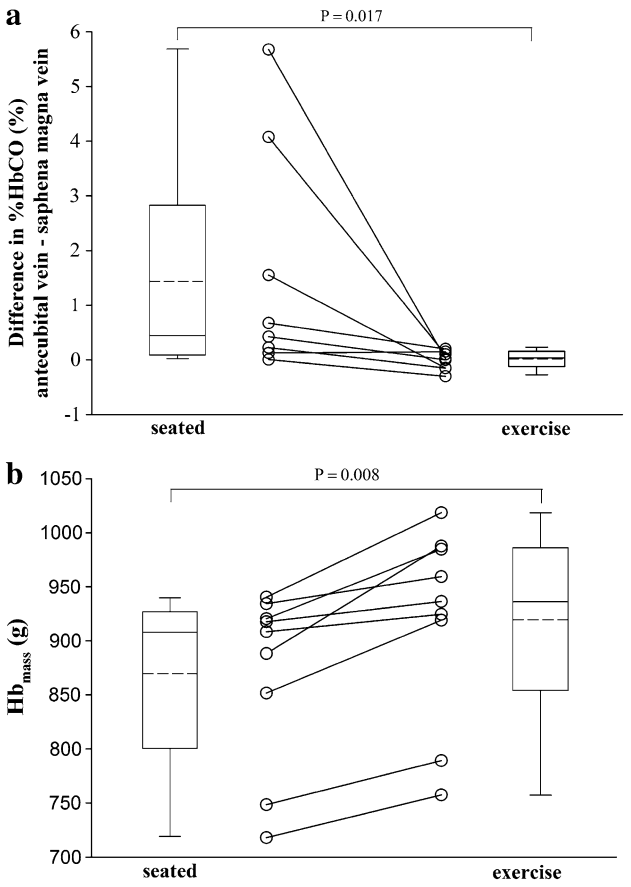

Fig. 1 **a** Difference in carboxyhemoglobin (%HbCO) in blood samples drawn from an antecubital and the saphena magna vein during the CO re-breathing procedure performed in the resting seated position and during light exercise. In the *box-plots*, the *dashed lines* indicate the mean and the *solid lines* the median scores. The *white points* illustrate the individual differences in %HbCO of every subject ($n = 8$). The difference in %HbCO between antecubital and saphena magna at rest varies greatly between subjects and may be related to vessel structure, degree of sympathetic constraint or different degrees of constraints to venous blood flow while seated. **b** Illustration of the total hemoglobin mass (Hb_{mass}) obtained during the resting seated position and with light cycle ergometer exercise when using antecubital vein blood samples. In the *box-plots* the *dashed lines* indicate the mean and the *solid lines* the median scores. The *white points* illustrates the individual Hb_{mass} of every subject ($n = 9$)

facilitate CO diffusion into the myocytes, but this remains speculative. A further limitation to our study is that we did not obtain %HbCO in the saphena magna vein before the re-breathing of CO, but assumed this value to be equal to %HbCO in the antecubital vein sample. It seems, however, very unlikely that differences between those two sampling sites in terms of %HbCO should exist in the resting human.

In conclusion, a CO re-breathing procedure performed in the resting seated position may underestimate Hb_{mass} due to uneven distribution of CO over the circulation, and we recommend adding light exercise to the CO re-breathing protocol. Future studies may attempt to decrease the re-breathing period if the CO is administered in combination with light exercise. Due to the higher blood flow in the pulmonary circulation re-breathing shorter periods may prove sufficient which would make the re-breathing procedure when combined with exercise easier to conduct. As an alternative to exercise it may be favorable to perform the CO re-breathing procedure in the complete supine position and perhaps in combination with passive movement of the extremities. Since strenuous exercise prior to the measurement of Hb_{mass} can influence the results (Robach et al. 2012) or to spuriously increase the values (Gough et al. 2012), it at present remains unknown if exercise prior to the measurement may be sufficient to facilitate a better CO distribution within the circulation.

Table 2 Total hemoglobin mass (Hb_{mass}, g), total hemoglobin mass expressed relative to body mass (Hb_{mass}/kg, g/kg), hemoglobin concentration ([Hb], g/dl), hematocrit (Htc, %), red cell volume (RCV, ml), plasma volume (PV, ml) and blood volume (BV, ml) for both CO re-breathing protocols

	Seated CO re-breathing $n = 9$	Exercise CO re-breathing $n = 9$	*P* values
Hb_{mass} (g)	908 (718–940)	936 (757–1018)	0.008
Hb_{mass}/kg (g/kg)	11.4 (10.3–13.3)	12.0 (10.8–14.3)	0.008
[Hb] (g/dl)	14.9 (13.5–15.9)	15.3 (14.2–16.2)	0.051
Hct (%)	43.8 (40.1–46.6)	45.9 (42.1–47.5)	0.050
RCV (ml)	2,670 (2,089–2,841)	2,862 (2,165–3,024)	0.008
PV (ml)	3,408 (2,436–4,180)	3,255 (2,802–4,155)	0.374
BV (ml)	6,094 (4,526–6,981)	6,065 (4,971–7,179)	0.374

Values are medians (minimum–maximum)

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Detection of blood volumes by means of CO re-breathing and Indocyanine Green and Sodium Fluorescein injections

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Running head: Comparison of different measurement methods for Hb_{mass} and blood volumes

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Abstract

By quantifying one blood volume compartment through administration of a specific tracer the remaining volumes can be calculated by the integration of venous haematocrit and/or haemoglobin concentration. However, since whole body haematocrit is higher than venous haematocrit such an approach might comprise certain errors. The main aim of the present study was to quantify the magnitude of errors introduced when estimating a given blood volume compartment through the direct determination of another compartment. For that purpose, different methods for detecting blood volumes and haemoglobin mass (Hb_{mass}) were compared, namely the carbon monoxide (CO) re-breathing (for Hb_{mass}), the Indocyanine green (ICG; for plasma volume (PV)) and the Sodium Fluorescein (SoF; for red blood cell volume (RBCV)) methods. No difference between ICG and CO re-breathing derived PV could be established when a whole body/venous haematocrit correction factor of 0.91 was applied (P=0.11, r=0.43, mean difference -340±612 ml). In contrast, when comparing RBCV derived by the CO re-breathing and the SoF method, the SoF method revealed lower RBCV values as compared to the CO re-breathing method (P<0.05, r=0.95, mean difference -728±184 ml). It needs to be mentioned that, compared to the ICG and the SoF methods, the typical error (%TE) and hence reliability of the CO re-breathing method was lower for all measured parameters. Therefore, estimating blood volume compartments by the direct assessment of another compartment could be considered as a suitable approach and especially the CO re-breathing method proved to be a feasible and accurate method due to its low measurement error.

Key words: Carbon monoxide, plasma volume, red blood cell volume, blood volume, haemoglobin mass, HbCO

Abbreviation list

BV, blood volume; PV, plasma volume; RBCV, red blood cell volume; hct, haematocrit; [Hb], haemoglobin concentration; Hb_{mass}, haemoglobin mass; ICG, indocyanine green; SoF, sodium fluorescein; CO, carbon monoxide; HbCO, carboxyhaemoglobin; %TE, percent typical error.

Summary statement

This study demonstrates that the CO re-breathing technique can be considered as a suitable method to establish plasma and blood volume, especially in situations requiring a high reliability.

Introduction

The assessment of blood volume (BV), plasma volume (PV) and/or red blood cell volume (RBCV) is important in a scientific and clinical context. Intravascular volumes can be determined by a variety of methods based on the dilution principle, i.e. the volume of any compartment is derived from the dilution of an administrated tracer that is confined to that specific compartment. Once one compartment volume is quantified then others can be calculated by integration of the haematocrit (hct) or haemoglobin concentration [Hb]. Nevertheless, as haematocrit does not incorporate the plasma that is bound to the endothelial surface layer of blood vessels (1), its use for the calculation of the unknown blood compartments is associated with inaccuracy. To overcome this limitation a factor (whole body hct/venous hct) of 0.91 has been implemented (2). Nevertheless, since in reality the ratio between whole body hct/venous hct varies from 0.73 to 1.10 (3), this correction factor represents merely a coarse estimate. The overall rationale for this study was to test the validity of calculating all blood compartments from a single measured compartment by assessing PV, RBCV and haemoglobin mass (Hb_{mass}) directly and in combination. We quantified Hb_{mass} by carbon monoxide (CO) re-breathing and PV and RBCV by injections of indocyanine green (ICG) and sodium fluorescein (SoF), respectively, to elucidate the below study questions.

Today the most commonly used approach for the determination of PV is the ICG dye method (4-7). ICG is a water soluble tracer, which rapidly binds to plasma proteins (mainly albumin). A unique feature of ICG is that it penetrates the endothelial surface layer (8, 9) and thus allows for determination of both circulating and non-circulating plasma. Thus, in an attempt to quantify the potential error of using a peripheral vein htc in combination with CO rebreathing determined Hb_{mass} for the estimation of PV and RBCV, we assessed PV directly by ICG and compared and contrasted these to the estimated PVs as derived from Hb_{mass} determinations. Similar comparisons have previously been performed by Evans blue and CO re-breathing (10-12) but it remains unknown if Evans blue enters the endothelial surface layer.

SoF binds to red blood cells and may hence be used in a dye fashion to determine RBCV. The first application of this method showed a good reliability (13) and when compared to Cr^{51} no difference in RBCV was observed (14). Since these initial efforts, the counting procedure of fluorescent cells has become automated by the use of flow-cytometry, and the variation of coefficient for repeated measurements is reported being ≈ 3 % (15). Today technical advances allows for even greater precision when determining fluorescent marked cells as compared to non-marked cells, which may reduce the variation even further. Although the SoF approach has been used in limited clinical settings (16), the technique has not become a standardized method suitable for research purposes, and one further aim with this study was to test the feasibility and reliability of this method for scientific purposes.

Quantification of Hb_{mass} requires a marker that is specific for the red blood cells, and usually compounds that interact with haemoglobin or the unique proteins of the red cell membrane are used. In scientific studies the most frequently used method is the CO re-breathing technique where haemoglobin bound CO (HbCO) is used as a marker. Two variants of the CO re-breathing method exist, the classic ten minute version (CO_{com}) (2) and a shortened protocol consisting of only two minutes of re-breathing (CO_{short}) (17). Whereas the CO_{com} has been successfully validated against gold standard radioactive ^{51}Cr labelling (10, 18) this is not the case for CO_{short}. We have attempted to further improve the procedure for the CO_{com} protocol by facilitating mixing of the administrated CO throughout the circulation by adding whole body tilting to the procedure ($CO_{com+tilt}$). This may reduce the typical error of measurement for Hb_{mass} to less than 1.5% (19). Whereas the CO_{short} may seem appealing due to its short period of rebreathing, this approach could indeed elicit inferior analytical results, due to among others reduced blood circulation time (20) and the necessity to estimate pulmonary CO loss to the atmosphere. Nonetheless, its typical error is similar to that of CO_{com} (17) and comparable results between the two approaches have been published (21). In the present study we wished to test if the above mentioned tilting procedure would be applicable to the CO_{short} also. Furthermore, since most exercise related interventions aiming at increasing Hb_{mass} only do so to a minor extent (22, 23) we wished to determine the precision of the CO re-breathing methods to detect a minute (3%) change in BV as induced by blood removal.

In summary, we had the following aims: i) To compare the ICG and the CO re-breathing method, ii) to compare the SoF and the CO re-breathing method, iii) to compare the common and the short CO re-breathing technique, iv) to elaborate on the influence of tilting on the CO re-breathing, v) to investigate HbCO kinetics during the CO re-breathing procedure, and vi) to test the reliability of the CO re-breathing methods by means of phlebotomy.

Methods

Participants

Twenty male subjects (24 ± 3 yrs, 78.3 ± 6.6 kg, 1.82 ± 0.05 m, mean ± SD) participated in this study. They were fully informed of the risks and discomforts associated with the experiments and oral and written informed consent was obtained prior to participation. All participants refrained from strenuous exercise on the day of the measurements and from alcohol and caffeine for 12 h prior to the tests. In addition, they refrained from blood donation and non-prescription drugs for the entire study duration. Individuals who had either donated blood or sojourned to above 2.500 m above sea level for more than 1 day within the last month before the study onset were excluded. All experimental protocols and procedures were approved by the cantonal ethics committee of Zurich (KEK-ZH-Nr. 2015-0014) and conformed to the Declaration of Helsinki.

Study design

All participants completed eight consecutive days of testing (figure 1). Over the first six study days intravascular volumes were determined by three different CO re-breathing approaches ($CO_{com+tilt}$, CO_{short}, $CO_{short+tilt}$). Each was conducted on two consecutive days (duplicate measurements) and the order of the three different approaches was randomised. In addition, 15 min after the $CO_{com+tilt}$ method either the ICG or SoF methods were applied in 10 subjects each.

On study day seven, whole blood corresponding to 3 % of the individual Hb_{mass} was withdrawn and discarded. Immediately after this, and at the same time on the following day, Hb_{mass} was determined with the $CO_{com+tilt}$ method (n = 10) or with the CO_{short} method (n = 10) to test the reliability of the two methods.

Experimental measures

Great care was given that measurements always took place at the same time of the day ± 2 h and eating and drinking habits were kept constant 6 h before the measurement throughout the entire study duration.

Common CO re-breathing ($CO_{com+tilt}$): This CO re-breathing protocol was based on the principles of Burge and Skinner (2) but included modifications (19). We have previously observed that gravitational blood pooling leads to inhomogeneous CO distribution over the intravascular space if CO re-breathing is performed in a seated position (20). To avoid this we facilitated venous return by passive whole body tilting. After participants were instrumented with a 20 gauge catheter (BD Venflon Pro Safety, Franklin, Lakes, NJ, USA) in an antecubital vein they were placed supine on a tilt table. Throughout the entire following procedure participants were alternatingly tilted to 15° head-down for 2 min and to 15° head-up for 1 min. First they breathed room air for 10 min. This was followed by a 4 min period where participants breathed 100 % oxygen from a Douglas bag. They were then switched by a sliding valve to a re-breathing circuit that was previously flushed with pure O_2. After confirmation of participants'

comfort a 1 ml venous blood sample was collected and analysed in triplicate for the fraction of carboxyhaemoglobin (HbCO) and haemoglobin concentration ([Hb]) on a hemoximeter (ABL800, Radiometer, Copenhagen, Denmark) and for haematocrit (Hct) by the micro-method (4 min at 13.500 rpm). Immediately afterwards 1.5 $ml.kg^{-1}$ bodyweight of 99.997 % chemically pure CO (CO N47, Air Liquide, Pullach, Germany) was injected into the re-breathing circuit. After 10 min of re-breathing another 1 ml blood sample from the arm was obtained and analysed as described above. Participants then performed a complete exhalation where after the re-breathing circuit was closed by the sliding valve and the participants disconnected. The volume of gas remaining in the re-breathing bag was quantified with a calibration pump and the CO content determined (Monoxor III, Bacharach Inc., New Kensington, PA, USA). Together with the previously measured dead space of the re-breathing circuit (980 ml) and the participants' predicted residual volume (24) this allowed calculation of the number of CO molecules that were not absorbed (nCO_{rem}).
This measure was exclusively performed by SK who at that point in time had determined Hb_{mass} on more than 300 occasions using this method (20, 25).

Short CO re-breathing (CO_{short} and $CO_{short+tilt}$): The short CO re-breathing protocol was conducted according to a slightly modified version (26) of the CO re-breathing procedure described by Schmidt and Prommer (17). Briefly, after the subjects had spent 10 min in a recumbent position a venous blood sample was drawn from an antecubital vein (BD Valu-Set, Franklin, Lakes, NJ, USA) and immediately analysed in triplicate for [Hb] on a hemoximeter (ABL800, Radiometer, Copenhagen, Denmark) and for haematocrit (hct) by the micro-method (4 min at 13.500 rpm). Afterwards participants were transferred to a chair. After 5 min in the sitting position, three capillary blood samples (35 µl) were taken from an earlobe and analysed immediately for HbCO (ABL800, Radiometer, Copenhagen, Demark). Then the participants where connected to a previously O_2 flushed glass spirometer (Blood Tec GbR, Bayreuth, Germany) including a 3.5 L anaesthetic bag filled with oxygen and inhaled a bolus of chemically pure CO (Multigas SA, Domdidier, Switzerland) corresponding to 1.2 $ml.kg^{-1}$ bodyweight with an upper limit of 100 ml. After inhaling the CO and the oxygen, participants held their breath for 10 s before they began re-breathing in the closed circuit for 1 min 50 s. After these two minutes participants performed a complete exhalation where after the re-breathing circuit was closed and the subject disconnected. At 6, 8 and 10 min after inhalation of the CO, three blood samples were taken from the earlobe and analysed as described above. To account for the CO exhaled between re-breathing termination and the withdrawal of the final blood samples, the difference between the end-tidal CO concentrations (Dräger PAC 7000, Dräger Safety, Lübeck, Germany) before and after the re-breathing procedure was multiplied by the estimated alveolar ventilation of 5.25 $l.min^{-1}$ (nCO_{exh}) (27). The volume of CO that has not been absorbed by the body was quantified by multiplication of the CO concentration in the anaesthetic bag (Dräger PAC 7000, Dräger Safety, Lübeck, Germany) with the system volume (spirometer volume + volume of the bag (3.5 l) and the estimated participant's residual volume (24)

(nCO_{rem}). To account for the loss of CO flux to myoglobin a correction factor of 0.3 %.min^{-1} of administered CO was applied (nCO_{myogl}) (28).

The procedure for the $CO_{short+tilt}$ method was the same as in the CO_{short} method but was conducted on a tilt table and included the same tilting regime as in the $CO_{com+tilt}$ (alternatingly 2 min 15° head-down and 1 min 15° head-up). First, participants were also placed on the tilt table and breathed room air while being tilted. Then the CO was injected and the tilting process continued until the last blood sample at min 10 was obtained.
Hb_{mass} was determined by ST in one half of the subjects using the CO_{short} and $CO_{short+tilt}$ method, whereas in the other half Hb_{mass} was measured by TS. Both investigators had performed more than 300 measurements, respectively with this method before conducting the present study (26, 29, 30).

Blood sampling from a foot vein: An additional catheter was inserted into a suitable vein at the dorsum of the foot during the first of the two measurements of each CO re-breathing method ($CO_{com+tilt}$, CO_{short}, $CO_{short+tilt}$) to evaluate the distribution of CO over the intravascular space by assessing the difference in HbCO between the foot and the antecubital vein or the earlobe, respectively, before and after the CO re-breathing.

Calculations: For the $CO_{com+tilt}$ method original Hb_{mass} was derived from equations 1 and 2:

$$nCO(mmol) = {(P_B/760) \times (vCO_{adm} - vCO_{rem})}/{r \times (273 + T)} \quad \text{eqn. 1}$$

$$Hb_{mass}(g) = {(nCO \times 25 \times 64.45)}/{\Delta HbCO} \quad \text{eqn. 2}$$

where P_B is the barometric pressure in mmHg, vCO_{adm} is the amount of CO that was administered in ml, vCO_{rem} is the amount of CO remaining in the re-breathing system in ml, r is the gas constant (0.08206), T is the temperature in °C, and ΔHbCO is the change in %HbCO between the first and second blood sample in %.

For the CO_{short} and $CO_{short+tilt}$, respectively, Hb_{mass} and PV parameters were calculated as described in equation 3:

$$Hb_{mass}(g) = {((k \times (vCO_{adm} - vCO_{rem} - vCO_{exh} - vCO_{myogl}) \times 100)}/{(\Delta HbCO \times 1.39)} \quad \text{eqn. 3}$$

where k = (P_B x 273)/760 x (273 + T), P_B is the barometric pressure in mmHg, T is the temperature in °C, vCO_{adm} is the amount of CO that was administered in ml, vCO_{rem} is the amount of CO remaining in the re-breathing system in ml, vCO_{exh} is the amount of CO which was exhaled between re-breathing termination and the withdrawal of the final blood samples in ml, vCO_{myogl} is the amount of CO flux to myoglobin in ml, ΔHbCO is the change in %HbCO between the blood samples taken before and after the CO re-breathing in %, and 1.39 is Hüfners number for the CO-binding capacity of haemoglobin.

Additionally, to overcome the differences in these original calculations, both calculations were also adjusted to each other by the inclusion/exclusion of the correction factor for CO flux to myoglobin and by matching the time-points of the withdrawal of the blood samples.

RBCV, BV and PV for $CO_{com+tilt}$ and CO_{short} and $CO_{short+tilt}$, respectively, were subsequently calculated from equations 4-6:

$$RBCV\ (ml) = {(Hb_{mass}\ \times hct)}/{[Hb]} \qquad \text{eqn. 4}$$

$$BV\ (ml) = {(RBCV\ \times 100)}/{(hct\ \times 0.91)} \qquad \text{eqn. 5}$$

$$PV\ (ml) = BV\ - RBCV \qquad \text{eqn. 6}$$

where 0.91 is the whole body/venous haematocrit correction factor.

Indocyanine green (ICG): PV was determined by the ICG method with some changes compared to previous protocols (5, 31). A set of ICG standards were prepared by mixing aliquots (250 µl) of plasma obtained from the investigated subject with known concentrations of ICG (0, 0.3125, 0.625, 1.25, 2.5 µg/ml final concentrations). These samples were transferred into micro-cuvettes (micro-UV-Cuvettes, 230-900 nm, BRAND, Germany) and the absorbances (abs) were determined in duplicates at 800 and 900 nm (UviLine 9100, SI analytics, Mainz, Germany). A standard curve was constructed by plotting the mean of the duplicate measures for each ICG standard versus its concentration in µg/ml. Plasma without ICG was blank. The standard curve was then used to determine the ICG concentration at the time point T_0 (see below). Prior to intra venous ICG injection subjects were seated on a rowing ergometer (Concept2 inc., Morrisville, VT, USA) where after a known amount of ICG (0.25 mg ICG/kg body weight; IC-Green, Akorn Inc., Lake Forest, IL, USA) was administrated by means of an intra venous bolus injection (time zero, T_0). Immediately there-after the subjects were instructed to start rowing (20 strokes/min and 2.5 m/sec, i.e. very light whole body exercise) and to maintain rowing until the last blood sample had been withdrawn. The intent herewith was to facilitate the mixing of ICG

throughout the circulation. Two minutes after injection (T_2) blood samples (~0.8 ml) were withdrawn every 20 s, up to 8 min (18 samples in total) and immediately transferred into Eppendorf tubes containing EDTA (1% final concentration). Samples were briefly centrifuged at 16'900 rcf (Eppendorf Centrifuge 5415 R, Eppendorf, Hamburg, Germany) and 120 µl plasma was transferred into a single use micro-cuvette and measured as described above. A semi-log graph was created by plotting the log(abs800-900 nm) measurement versus time (in min). The one phase decay function within the nonlinear regression function in GraphPad Prism (6.0, GraphPad Software, Inc., La Jolla, CA, USA) was applied to calculate T_0. Outliers were identified by an algorithm within the software. Finally, PV was estimated from distribution space of ICG (DS_{ICG}) from equation 7:

$$PV \approx DS_{ICG} = {}^{D}/_{CP_0} \qquad \text{eqn. 7}$$

Where D is the amount (in mg) of injected dye and CP_0 is the calculated plasma ICG concentration at T_0. RBCV, BV and Hb_{mass} were subsequently calculated according to eqn. 4-6.

Sodium fluorescein (SoF): RBCV was estimated by labeling a known number of red blood cells (RBC) with the fluorescent dye sodium fluorescein (Fluoresceine 10% Faure, Curatis AG, Liestal, Switzerland) (RBC_F) where after the RBC_F were re-injected into the circulation and its fraction determined by flow cytometry (16, 31). Briefly, 20 ml blood was withdrawn and labeled with fluorescein. For this the blood was centrifuged for five minutes at 1800 rcf, brake off (Eppendorf Centrifuge 5702R, Eppendorf, Hamburg, Germany) and the plasma removed and discarded. The remaining RBC were incubated with 48 mg SoF (6 ml) and gently mixed for five minutes. Access dye was removed by 2 times washing with a Ca-Gluconate solution (Calcium-Sandoz 10%, Sandoz Pharmaceuticals GmbH, Holzkirchen, Germany) and the final volume was adjusted to 20 ml with Ringer's lactate (Bichsel AG, Interlaken, Switzerland). The RBC_F-suspension was the administrated into the subject´s circulation by an intra venous bolus injection (time zero, T_0) where after rowing was initiated as stated for the ICG trial. Four (T_4), 6 (T_6) and 8 (T_8) min after injection 1 ml of blood was drawn and stored on ice. Immediately after the last sample was obtained, the fraction of RBC_F in each sample was determined in triplicate by flow cytometry (FACSCanto, BD Biosciences, San Jose, CA, USA). RBCV was calculated according to equation 8:

$$RBCV = {(hct_s \times V_s \times hct_{vb})}/{(hct_{cir} \times F_{RBCF})} \qquad \text{eqn. 8}$$

where, hct_s is the haematocrit of the injected RBC_F-suspension, V_s is the volume of injected RBC_F-suspension, hct_{vb} is the haematocrit before injection of the RBC_F-suspension, hct_{cir} is the average

haematocrit in the blood samples at T_4, T_6 and T_8 and F_{RBCF} is the average fraction of RBC_f in the blood samples at T_4, T_6 and T_8. BV, PV and Hb_{mass} were subsequently calculated according to eqn. 4-6.

Phlebotomy: First, a venous catheter was placed as described above. After a 20 min resting period a first blood sample (1 ml) was taken and analysed for hct and [Hb]. These values, together with the Hb_{mass} determined from the previous CO re-breathings ($CO_{com+tilt}$ or CO_{short}), allowed for calculation of the total amount of blood which had to be removed for a 3 % reduction in Hb_{mass}. After phlebotomy, and as soon as participants felt comfortable and ready, the planned CO re-breathings ($CO_{com+tilt}$ or CO_{short}) were performed.

Statistical analysis

Statistical evaluation of the data was performed by using the software SAS Enterprise Guide (4.3, SAS Institute, Inc., Cary, 220 NC, USA). The significance level was set at $P < 0.05$ and the data is presented as mean ± SD, unless otherwise indicated. Comparison of the different methods was made using a one way repeated measurements analysis of variance (ANOVA) with the method (e.g. $CO_{com+tilt}$, CO_{short}, $CO_{short+tilt}$) as main effect. Tukey's range test was applied for post hoc analysis. Single comparisons were performed using a paired t-test. For HbCO analysis statistics for unpaired values was used. P-values are presented together with effect-sizes described using Cohen's d (with $d \leq 0.2$ representing a trivial difference; 0.2 – 0.5, a small difference; 0.5 – 0.8 a moderate difference; and > 0.8 a large difference). To visualise the differences between the different methods the Bland-Altman plot was used (32). Bivariate associations were determined by Pearson's Correlation Coefficients where $r = 0.1$ represents a small, $r = 0.3$ a moderate and $r = 0.5$ a large correlation. The coefficient of variation for the different parameters was expressed as the typical error, which is calculated as the standard deviation of difference scores divided by $\sqrt{2}$ (33). When expressed as a percent of the mean, the percent typical error (%TE) is obtained (33). Effect sizes and %TE are presented along with the 95 % likely range of the true value [95 % confidence interval].

Results

Blood volumes as determined by ICG and compared to $CO_{com+tilt}$

In this study, the %TE [95 % CI] (n = 10) for Hb_{mass}, RBCV, PV and BV when derived from the ICG method were 11.23 [6.69; 18.88], 12.15 [7.23; 20.41], 8.70 [5.18; 14.62] and 9.99 [5.95; 16.78], respectively. The respective values when determined by the $CO_{com+tilt}$ method (n = 20) were 1.19 [0.71; 2.00], 1.60 [0.95; 2.69], 3.57 [2.13; 6.00] and 2.19 [1.30; 3.68].

All Hb_{mass} and blood volume values as determined by the $CO_{com+tilt}$ and ICG methods are given in table 1. Briefly, PV as determined with the ICG method was not different than PV as estimated from the $CO_{com+tilt}$ method (-7.0 ± 12.2 %, P = 0.11, d = 0.59 [-0.30; 1.49], mean difference -340 ± 612 ml, and its values are illustrated in a Bland-Altman plot (Figure 2A). For PV no correlation (P = 0.21, r = 0.43) between the ICG and the $CO_{com+tilt}$ methods could be established. Also in all the other determined parameters no differences were apparent.

Blood volumes as determined by SoF and compared to $CO_{com+tilt}$

In this study, the %TE obtained when using the SoF method (n = 10) were 5.96 [3.55; 10.01] for Hb_{mass}, 5.97 [3.55; 10.03] for RBCV, 7.95 [4.73; 13.36] for PV and 6.95 [4.14; 11.68] for BV.

As presented in table 1, SoF determined RBCV revealed a lower RBCV when compared to values based on the $CO_{com+tilt}$ method (-27.8 ± 3.6 %, P < 0.01, d = 1.88 [0.83; 2.93], mean difference (-728 ± 184 ml). The measured differences in RBCV between the SoF and the $CO_{com+tilt}$ methods are presented in a Bland-Altman plot (Figure 2B). Correlational analysis revealed an association (P < 0.01, r = 0.95) between SoF and $CO_{com+tilt}$ derived RBCV. Also all the other measured parameters were lower in the SoF method as compared to the $CO_{com+tilt}$ method.

Comparison of the different CO re-breathing methods

The %TE for blood volumes determined by the $CO_{com+tilt}$ method are presented above. The respective values for Hb_{mass}, RBCV, PV and BV were 1.93 [1.15; 3.24], 3.03 [1.80; 5.09], 2.69 [1.60; 4.52] and 1.84 [1.10; 3.09] for the CO_{short} method (n = 20) and 1.31 [0.78; 2.20], 2.69 [1.60, 4.52], 3.17 [1.89; 5.33] and 2.41 [1.43; 4.05] for the $CO_{short+tilt}$ method (n = 20).

Hb_{mass} and blood volumes of all three CO re-breathing methods are presented in table 2. In $CO_{com+tilt}$ Hb_{mass} was lower (both P < 0.01) compared to CO_{short} (-4.6 ± 2.4 %, d = 0.35 [-0.28; 0.97], mean difference -43 ± 23 g) and $CO_{short+tilt}$ (-2.1 ± 2.5 %, d = 0.15 [-0.47; 0.74], mean difference -18 ± 23 g). When comparing CO_{short} with $CO_{short+tilt}$, CO_{short} revealed a +24 ± 21 [15; 33] g (2.7 ± 2.4 %) higher (P < 0.01, d = 0.20 [-0.42; 0.83]) Hb_{mass} than $CO_{short+tilt}$.

Figure 3 illustrates the individual differences between Hb_{mass} as determined by the different CO re-breathing techniques in Bland-Altman plots. Correlational analysis revealed a large association ($P < 0.01$) between Hb_{mass} derived from all three approaches. Pearson correlation coefficient was 0.98 for $CO_{com+tilt}$ vs. CO_{short} and for $CO_{com+tilt}$ vs. $CO_{short+tilt}$ and 0.96 for CO_{short} vs. $CO_{short+tilt}$.

The influence of different calculation options on Hb_{mass} are given in table 3. Briefly, independent of sampling points, Hb_{mass} was lower ($P < 0.01$) when a theoretical based correction factor for CO bound to myoglobin was applied. Furthermore, in CO_{short} and $CO_{short+tilt}$ Hb_{mass} was higher ($P < 0.01$) when the calculations were based on blood samples obtained at min 8 and 10 compared to min 6 and 8 and reached its highest values ($P < 0.05$) when only the 10 min samples were used for the calculations.

HbCO kinetics

It proved challenging to obtain blood samples from a vein at the dorsum of the foot in some study participants and only volunteers with a complete set of data were included into this analysis ($n = 15$ for $CO_{com+tilt}$, $n = 12$ for CO_{short}, and $n = 10$ for $CO_{short+tilt}$).

ΔHbCO values from pre re-breathing to min 6, 8 and 10 from the arm/ear and the foot and their difference (arm/ear-foot) for all three CO re-breathing techniques are presented in table 4. The difference in ΔHbCO between the arm/ear and the foot gradually decreased ($P < 0.01$) from min 6 to min 10. At min 10 the arm/ear value was not different from the foot value for $CO_{com+tilt}$ ($P = 0.55$, $d = 0.21$ [-0-50; 0.93], mean difference +0.20 ± 0.42 %), CO_{short} ($P = 0.09$, $d = 0.72$ [-0.10; 1.55], mean difference 0.73 ± 1.38 %) and $CO_{short+tilt}$ ($P = 0.22$, $d = 0.57$ [-0.32; 1.47], mean difference +0.46 ± 0.60 % and also their mean differences (arm/ear-foot) did not differ from each other ($P = 0.22$, $d = 0.55$ [-0.23; 1.32] for $CO_{com+tilt}$ vs. CO_{short}, $P = 0.57$, $d = 0.25$ [-0.60; 1.09] for CO_{short} vs. $CO_{short+tilt}$ and $P = 0.21$, $d = 0.52$ [-0.29; 1.34] for $CO_{com+tilt}$ vs. $CO_{short+tilt}$). Individual data of the difference of ΔHbCO between the arm/ear and the foot at min 6, 8 and 10 are illustrated in figure 4.

Phlebotomy

For phlebotomy 183 ± 28 and 195 ± 24 ml of whole blood was removed for the $CO_{com+tilt}$ and the CO_{short}, respectively. This corresponded to 26 ± 4 and 28 ± 3 g of haemoglobin which equalled 3 % of the participants Hb_{mass}. After phlebotomy the measured mean difference in Hb_{mass} for the $CO_{com+tilt}$ was 27 ± 15 g (3.1 ± 1.6 %) and for the CO_{short} 28 ± 16 g (3.0 ± 1.5 %), which was not statistically different from the actual removed amount of blood ($P = 0.89$, $d = 0.07$ [-0.58; 0.72] and $P = 0.98$, $d = 0.01$ [-0.64; 0.65]). The measured difference in Hb_{mass} from pre to post phlebotomy ranged from 1 to 51 g (0.1 to 5.1 %) in the $CO_{com+tilt}$ and from -4 to 53 g (-0.5 to 4.7 %) in the CO_{short}.

Discussion

The aim of this study was to compare common methods for the quantification of blood compartment volumes and Hb_{mass} and to determine whether one blood compartment can be estimated by quantification of another compartment. The main findings are that i) PV estimated by the $CO_{com+tilt}$ method was not different from PV measured by ICG, but associated with a higher reliability, ii) RBCV measured by SoF differed from RBCV when estimated from $CO_{com+tilt}$ and furthermore proved to be less reliable, iii) although the reliability of all CO re-breathing methods was similar and correlations between methods were high, the measured Hb_{mass} differed between the three methods, iv) although tilting facilitated CO distribution throughout the circulation, Hb_{mass} was lower in the tilted protocol as compared to the seated version, v) the difference between ΔHbCO between the arm/ear and the foot blood sample continuously decreased with time and was similar at min 10, and vi) both, the common and the short CO re-breathing, showed a high reliability and were able to detect a 3 % change in Hb_{mass}.

ICG vs. CO re-breathing

With the $CO_{com+tilt}$ method only Hb_{mass} is directly measured. Blood compartment volumes such as PV may subsequently be calculated by the integration of hct and [Hb] (19, 25), but this may be associated with errors as a peripheral venous htc may not reflect the whole body htc (34, 35). Since plasma bound to the endothelial surface layer will not be included in the CO re-breathing derived PV the ICG method is expected to yield higher PV results than the $CO_{com+tilt}$ method unless the difference in whole body to venous haematocrit is corrected for. The application of a correction factor of 0.91 has become widely accepted for the calculation of PV, although actual values range from 0.73 to 1.10 (3). With a correction factor of 0.91 (2), the $CO_{com+tilt}$ yielded a 340 ml higher PV value than the ICG method which, however, was not statistically significant. In contrast, without application of the correction factor, PV derived from $CO_{com+tilt}$ would have been 293 ml lower than that provided by the ICG method. This difference is within the expected range of endothelial surface layer bound PV (5). In the present study the application of a correction factor of 0.96 would have resulted in a mean PV difference of only 27 ml between the two methods, and this value could be considered to be used in future studies. However, it needs to be acknowledged that this is only true for the calculation used in this study. Different formulas to calculate PV from Hb_{mass} exist (36) and depending on the calculations likely also the correction factor needs to be adapted. Furthermore, it should be mentioned that individual mean difference showed a high variation between subjects. Indeed, although average PV assessed by the two methods was similar, no correlation (P = 0.21, r = 0.43) was found between individual values obtained by the two methods. Notably, the %TE for ICG determined PV was more than double that for the $CO_{com+tilt}$ method and to our knowledge such low %TE (3.57 %) has not been reported for any method used to determine PV.

Although the ICG method has been considered as one of the best methods for measuring plasma volume the method has limitations. These are mainly related to the applied back extrapolation, which is

influenced by blood mixing time (31, 37, 38). Accordingly, Polidori and Rowley (39) suggest not to back extrapolate to the time of ICG injection (t_0) but to t_1 since back extrapolating to t_0 would underestimate PV. Complete mixture was initially suggested to occur within 2 min (6), although mixing time varies between subjects (40). However, more recent experiments suggested that full mixing requires 3-5 min (38). To overcome the variations induced by incomplete blood mixing we accelerated mixing with light rowing exercise since an increased cardiac output has been shown to minimize the error of back extrapolation (40).

Blood volumes as determined by SoF and compared to $CO_{com+tilt}$

Despite a very good correlation ($r = 0.95$, $P < 0.01$) between the SoF and $CO_{com+tilt}$ method, mean RBCV was 728 ± 114 ml lower ($P < 0.01$) in the SoF trials. The reason for this difference remains unclear. However, while the actual volumes are unknown, the SoF based values are at the lower end of what can be expected in healthy young men whereas the values from the $CO_{com+tilt}$ method are within the normal range. In the present study the %TE for SoF determined RBCV was 5.97 % whereas the %TE of the $CO_{com+tilt}$ was 1.6 %. Although previous studies have reported a %TE of ~3 % for the SoF method (15, 41) the SoF approach seems unfeasible for the detection of small changes/differences in RBCV.

The advantage of this method is that SoF has a half-life < 0.5 h, so that measurements can be repeated after just 1 h (15). Furthermore, unlike with the CO re-breathing methods, no subject cooperation is required and measurements can hence be performed in unconscious humans, which is appealing for clinical settings. On the other hand, the experiments are time consuming and the analytical results are not available within 1 h (16). Furthermore, trained personnel is required to perform the preparing, labelling and washing of the red blood cells.

Comparison of the three CO re-breathing methods

Although a strong correlation ($r = 0.98$, $P < 0.01$) was found between $CO_{com+tilt}$ and CO_{short}, Hb_{mass} was 43 ± 23 g lower when determined with $CO_{com+tilt}$. In contrast, previous studies have found no difference (17) between the common and the short CO re-breathing or even a slightly higher Hb_{mass} for the common version (21, 29, 42). The higher Hb_{mass} for the common version in the latter studies could however be explained by the inclusion of a correction factor for the hypothetical loss of CO to myoglobin, which is usually only performed in the short version (21, 29). Additionally, it remains controversial when the post CO re-breathing blood samples should be collected in CO_{short}. While blood is often sampled after 6 and 8 min, the recommendations vary between 5 and 10 min (17, 21, 28, 42). In our study we eliminated both bias by adjusting the inclusion of a theoretical loss of CO to myoglobin for all calculations and by obtaining blood samples at the same time point. However, while these differences might explain the lower values for Hb_{mass} in CO_{short} in the above mentioned references, the adjustment of these concerns only further increased the difference between $CO_{com+tilt}$ and CO_{short} (~100 g) in our study.

A difference between the common and the short re-breathing protocols in the present study was the volume of administered CO. Whereas in the CO_{short} and $CO_{short+tilt}$ procedure 1.2 $ml.kg^{-1}$ body weight of CO was administered it was 1.5 $ml.kg^{-1}$ body weight in the $CO_{com+tilt}$ procedure. We did not standardize this since we intended to compare the two procedures in the way they are normally conducted. Turner et al. compared a CO bolus of 1.0 and 1.4 $ml.kg^{-1}$ using the CO_{short} procedure and found a 12 g lower Hb_{mass} for the higher dose (43). A higher HbCO value can be measured with higher accuracy and therefore a ΔHbCO of 6.5 % should be aimed for (2). With progressively lower doses of CO, a 0.1 % difference in ΔHbCO is associated with a substantial increase in the measurement error (18). For the $CO_{com+tilt}$ procedure mean ΔHbCO was higher (8.1 %) than for the CO_{short} (6.0 %) and the $CO_{short+tilt}$ (6.1 %) procedure, but whether this is the reason for the discrepancies remains unknown. It should however be acknowledged that the %TE was similar in both procedures suggesting an equal reliability to detect Δ changes in Hb_{mass} as will also be discussed in the below.

The most obvious explanation for the lower Hb_{mass} in the common CO re-breathing is the inclusion of whole body tilting in $CO_{com+tilt}$. Although we did not conduct the common CO re-breathing in the seated position, indication that the tilting procedure might lead to a lower Hb_{mass} was given by results of the $CO_{short+tilt}$ trial which, against our hypothesis, revealed a 24 g lower Hb_{mass} compared to the CO_{short} trial. Apparently, although a more uniform blood mixing could be achieved (indicated by the HbCO comparison between the foot and the arm/ear as discussed in the below), a lower Hb_{mass} was obtained when the re-breathing procedure was combined with passive tilting.

HbCO kinetics

We have previously demonstrated that if the CO re-breathing is performed in a seated position, CO is not evenly distributed throughout the circulation (20) leading to an underestimation of Hb_{mass}. In the current study the CO re-breathing was combined with passive tilting to facilitate blood mixing. Indeed, as suggested by the lower difference in ΔHbCO between the arm/ear and foot samples in the $CO_{com+tilt}$ and the $CO_{short+tilt}$ as compared to the CO_{short} method, the tilting improved the distribution of CO throughout the circulation. The difference in ΔHbCO between the arm/ear and the foot blood sample gradually decreased from min 6 to 10 in both short procedures. This implies that for the shortened procedure, as for the common procedure, in some subjects blood mixing is not complete at min 6 and therefore blood samples should be taken at min 8 and 10 instead of min 6 and 8 as suggested by others (21, 28, 42). This however eliminates the "time optimization" of the shortened procedure.

Reliability of the CO re-breathing methods

To emphasize the high reliability of the CO re-breathing method indicated by the small %TE, the reliability of the $CO_{com+tilt}$ and the CO_{short} method was also experimentally tested by means of phlebotomy. Earlier studies have successfully demonstrated a close agreement between the measured

and calculated losses of Hb_{mass} when removing 500 ml of whole blood (2, 17). In the current study we removed only ~200 ml whole blood (3% of Hb_{mass}), and demonstrated that the CO rebreathing methods are capable of tracking such changes also.

Conclusion

This study was designed to compare different methods for measuring blood volumes and Hb_{mass}. Despite some deviations, the CO re-breathing method is suitable for determination of PV, especially in situations where a high reliability is needed.
Furthermore, the SoF approach is deemed less feasible for scientific studies than the CO re-breathing approaches. Finally, the applied CO re-breathing methods highly correlated to each other and demonstrated an excellent reliability albeit in absolute terms they differed somewhat.

Clinical perspectives

In some clinical settings the determination of blood volumes is crucial and often a different method for every blood volume in question is applied. Theoretically, if one blood compartment is quantified then the remaining volumes can be estimated by the integration of venous haematocrit (hct) or haemoglobin concentration. However, since whole body hct is higher than venous hct such an approach might comprise certain errors. Our findings, however, suggest that this error is negligible and that one blood compartment can be estimated by quantification of another compartment if a precise and reliable method such as the CO re-breathing is applied. To lower expenses and save time it could hence be considered to include only one method for detecting all blood volume compartments.

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Disclosure

None of the authors has any conflict of interest to declare.

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Table 1. Overview about hemoglobin mass (Hb_{mass}) and blood volumes of the common CO re-breathing ($CO_{com+tilt}$), the Indocyanine Green (ICG) and the Sodium Fluorescein (SoF) method.

	$CO_{com+tilt}$	ICG	SoF
ICG Group			
Hb_{mass} [g]	895 ± 84	886 ± 133	-
RBCV [ml]	2654 ± 248	2625 ± 395	-
PV [ml]	4385 ± 614	4045 ± 527	-
BV [ml]	7040 ± 831	6670 ± 887	-
SoF Group			
Hb_{mass} [g]	883 ± 154	-	636 ± 102**
RBCV [ml]	2606 ± 451	-	1878 ± 310**
PV [ml]	4093 ± 665	-	2944 ± 405**
BV [ml]	6701 ± 1074	-	4822 ± 682**

$CO_{com+tilt}$, common CO re-breathing on tilt table; ICG, Indocyanine Green; SoF, Sodium Fluorescein; Hb_{mass}, total hemoglobin mass; RBCV, red blood cell volume; PV, plasma volume; BV, total blood volume. Values are mean ± SD. *$P < 0.05$ and **$P < 0.01$ vs. $CO_{com+tilt}$. $n = 10$.

Table 2. Overview about hemoglobin mass (Hb_{mass}) and blood volumes of all three CO re-breathing methods ($CO_{com+tilt}$, CO_{short}, $CO_{short+tilt}$).

	$CO_{com+tilt}$	CO_{short}	$CO_{short+tilt}$
Hb_{mass} [g]	889 ± 121††§§	931 ± 121**§§	907 ± 114**††
RBCV [ml]	2631 ± 355††§§	2803 ± 341**§§	2695 ± 318**††
PV [ml]	4239 ± 641†§§	4041 ± 602*§§	3847 ± 520**††
BV [ml]	6870 ± 951§§	6844 ± 895§§	6542 ± 802**††

$CO_{com+tilt}$, common CO re-breathing on tilt table; CO_{short}, short CO re-breathing; $CO_{short+tilt}$, short CO re-breathing on tilt table; Hb_{mass}, total hemoglobin mass; RBCV, red blood cell volume; PV, plasma volume; BV, total blood volume. Values are mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. $CO_{com+tilt}$, $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$ vs. CO_{short}, $^{\S}P < 0.05$ and $^{\S\S}P < 0.01$ vs. $CO_{short+tilt}$. $n = 20$.

Table 3. Overview about CO re-breathing derived hemoglobin mass (Hb_{mass}) at different blood sampling points (Minute 6+8, Minute 8+10, Minute 10) and with or without a correction factor of CO bound to Myoglobin (Mb).

	$CO_{com+tilt}$	CO_{short}	$CO_{short+tilt}$
Minute 6+8§			
With Mb**	-	**931 ± 121**	**907 ± 114**
Without Mb	-	952 ± 123	927 ± 116
Minute 8+10††,§			
With Mb**	-	955 ± 123	916 ± 119
Without Mb	-	983 ± 126	942 ± 122
Minute 10††			
With Mb**	862 ± 117	963 ± 119	918 ± 121
Without Mb	**889 ± 121**	994 ± 123	948 ± 125

$CO_{com+tilt}$, common CO re-breathing on tilt table; CO_{short}, short CO re-breathing, $CO_{short+tilt}$, short CO re-breathing on tilt table; Hb_{mass}, total hemoglobin mass; Mb, Myoglobin. Values are mean ± SD. $^{**}P < 0.01$ compared to without Mb, $^{\dagger\dagger}P < 0.01$ vs. minute 6+8, $^{\S}P < 0.05$ and $^{\S\S}P < 0.01$ vs. minute 10. $n = 20$.

Table 4. ΔHbCO values from pre to minute 6, 8 and 10 from the arm or the ear, the foot and their difference (arm/ear-foot) for all three CO re-breathing methods ($CO_{com+tilt}$, CO_{short}, $COshort+tilt$).

	ΔHbCO pre-minute 6			ΔHbCO pre-minute 8			ΔHbCO pre-minute 10		
	ear	foot	difference	ear	foot	difference	arm/ear	foot	difference
$CO_{com+tilt}$	-	-	-	-	-	-	8.02±0.86	7.83±0.92	0.20±0.42
CO_{short}	6.27±0.57	4.08±2.22**	2.18±2.38	5.98±0.46	4.78±1.66*	1.2±1.73	5.81±0.44	5.08±1.36	0.73±1.38
$CO_{short+tilt}$	5.97±0.65	4.76±1.57*	1.21±1.44	5.90±0.62	5.1±1.22	0.7±0.95	5.84±0.64	5.38±0.94	0.46±0.60

$CO_{com+tilt}$, common CO re-breathing on tilt table; CO_{short}, short CO re-breathing; $CO_{short+tilt}$, short CO re-breathing on tilt table. Values are mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ arm/ear vs. foot. $n = 15$ for $CO_{com+tilt}$, $n = 12$ for CO_{short} and $n = 10$ for $CO_{short+tilt}$.

Figure legends

Figure 1. Schematic illustration of the study design. $CO_{com+tilt}$, common CO re-breathing in a tilted position; CO_{short}, short CO re-breathing in a seated position; $CO_{short+tilt}$, short CO re-breathing in a tilted position; ICG, indocyanine green method; SoF, sodium fluorescein method. Black arrow indicates an additional venous catheter in *V. Saphena Magna* for comparison between the blood in the foot and the arm or the earlobe, respectively. n = 20 for all CO re-breathing methods except for day 7 and 8 where n = 10 for each method. For the ICG and the SoF method n = 10 for each method.

Figure 2. Bland-Altman plot for A) the plasma volume (PV) between the common CO re-breathing method in a tilted position and the Indocyanine Green (ICG) method, and B) the red blood cell volume (RBCV) between the common CO re-breathing method in a tilted position and the Sodium Fluorescein (SoF) method. Solid horizontal lines indicate the mean difference between two measurements and dashed horizontal lines indicate 95 % limits of agreement. n = 10 for each method.

Figure 3. Bland-Altman plot for hemoglobin mass (Hb_{mass}) between A) the common CO re-breathing in a tilted position ($CO_{com+tilt}$) and the short CO re-breathing in a seated position (CO_{short}), B) the short CO re-breathing in a seated position (CO_{short}) and the short CO re-breathing in a tilted position ($CO_{short+tilt}$) and, C) the common CO re-breathing in a tilted position ($CO_{com+tilt}$) and the short CO re-breathing in a tilted position ($CO_{short+tilt}$). Solid horizontal lines indicate the mean difference between two measurements and dashed horizontal lines indicate 95 % limits of agreement. n = 20 for each method.

Figure 4. Individual data points of the ΔHbCO values from pre to minute 6, 8 and 10 from the difference between the arm or the ear, respectively, and the foot for all three CO re-breathing methods. The grey squares (■) represent the common CO re-breathing in a tilted position ($CO_{com+tilt}$), the white circles (○) represent the short CO re-breathing in a seated position (CO_{short}) and the black triangles (▲) represent the short CO re-breathing in a tilted position ($_{COshort+tilt}$). n = 15 for $CO_{com+tilt}$ (■), n = 12 for CO_{short} (○) and n = 10 for $CO_{short+tilt}$ (▲).

Figure 1

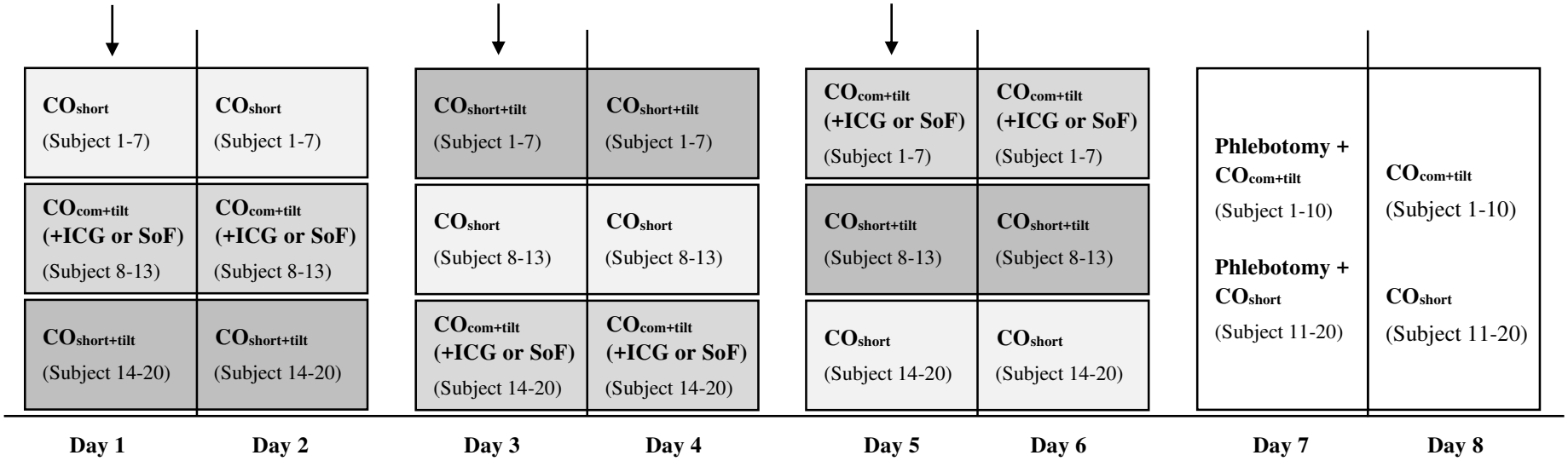
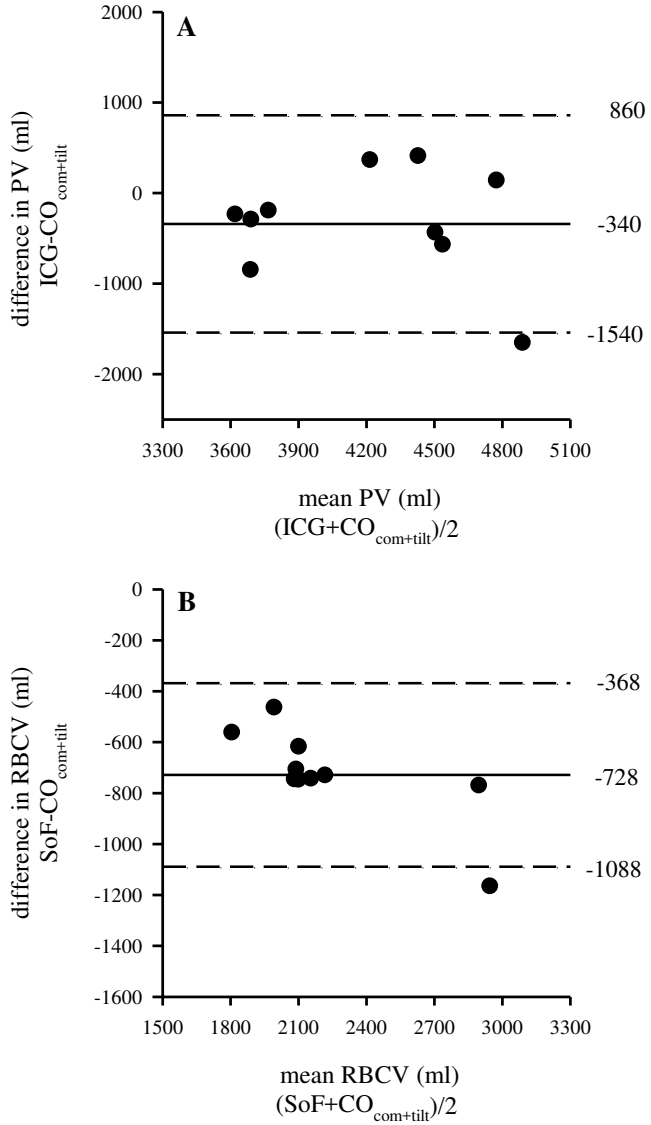

COshort (Subject 1-7)
COshort (Subject 1-7)
COcom+tilt (+ICG or SoF) (Subject 8-13)
COcom+tilt (+ICG or SoF) (Subject 8-13)
COshort+tilt (Subject 14-20)
COshort+tilt (Subject 14-20)
COshort+tilt (Subject 1-7)
COshort+tilt (Subject 1-7)
COshort (Subject 8-13)
COshort (Subject 8-13)
COcom+tilt (+ICG or SoF) (Subject 14-20)
COcom+tilt (+ICG or SoF) (Subject 14-20)
COcom+tilt (+ICG or SoF) (Subject 1-7)
COcom+tilt (+ICG or SoF) (Subject 1-7)
COshort+tilt (Subject 8-13)
COshort+tilt (Subject 8-13)
COshort (Subject 14-20)
COshort (Subject 14-20)
Phlebotomy + COcom+tilt (Subject 1-10)
Phlebotomy + COshort (Subject 11-20)
COcom+tilt (Subject 1-10)
COshort (Subject 11-20)
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8

Figure 2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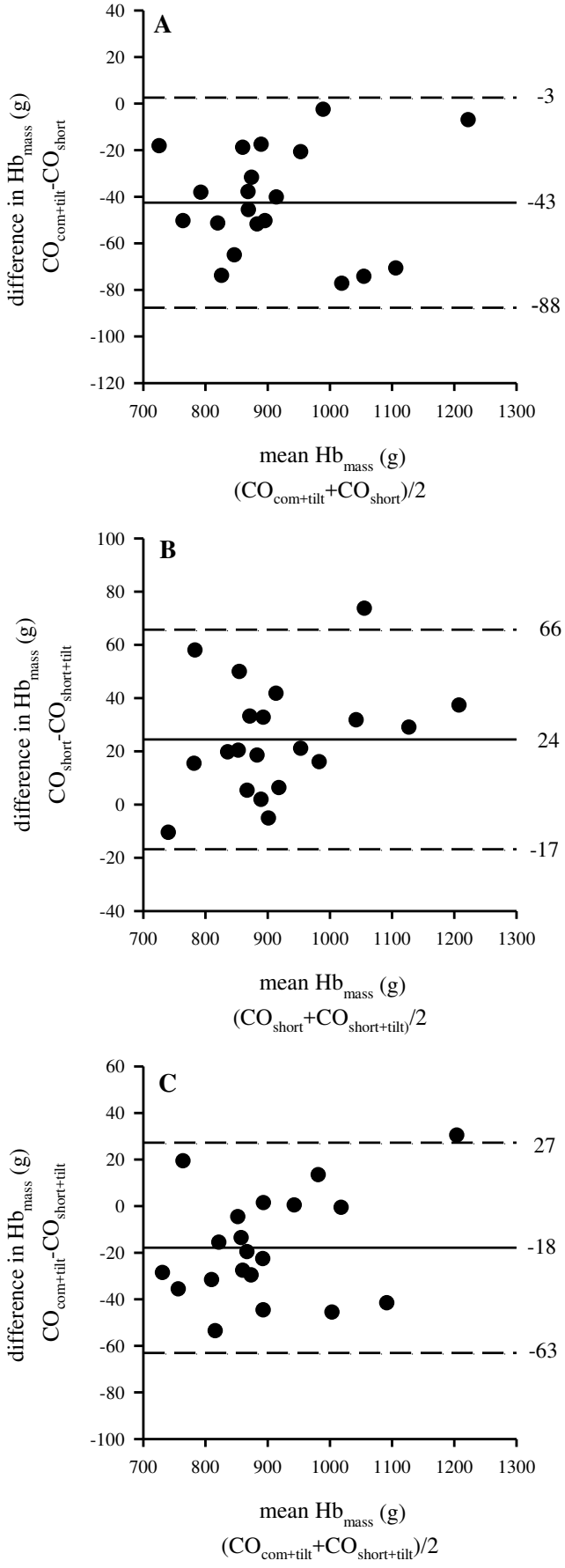
2000
1000
0
-1000
-2000
860
-340
-1540
3300
3600
3900
4200
4500
4800
5100
difference in PV (ml)
ICG-CO com+tilt
mean PV (ml)
(ICG+CO com+tilt)/2
B
0
-200
-400
-600
-800
-1000
-1200
-1400
-1600
-368
-728
-1088
1500
1800
2100
2400
2700
3000
3300
difference in RBCV (ml)
SoF-CO com+tilt
mean RBCV (ml)
(SoF+CO com+tilt)/2

Figure 3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-88
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1200
1300
difference in Hbmass (g)
COcom+tilt-COshort
mean Hbmass (g)
(COcom+tilt+COshort)/2
B
100
80
60
40
20
0
-20
-40
66
24
-17
difference in Hbmass (g)
COshort-COshort+tilt
mean Hbmass (g)
(COshort+COshort+tilt)/2
C
60
40
20
0
-20
-40
-60
-80
-100
27
-18
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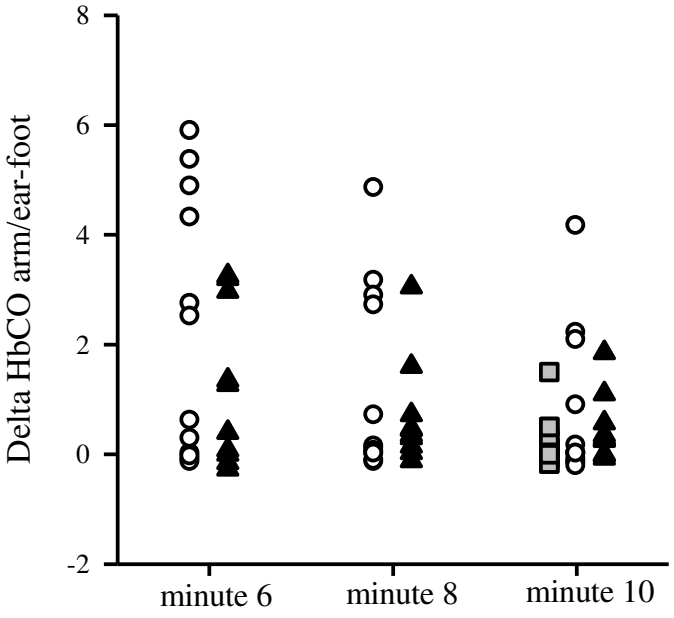
difference in Hbmass (g)
COcom+tilt-COshort+tilt
mean Hbmass (g)
(COcom+tilt+COshort+tilt)/2

Figure 4



3. Discussion and outlook

The purpose of the present PhD project was to enhance our understanding of the interaction between hyperthermia and aerobic exercise performance. Specifically, we examined whether hyperthermia-induced reductions in cerebral perfusion would limit exercise performance by centrally mediated fatigue. Furthermore, we focused on HA induced physiological adaptations and examined their effect on exercise performance in hot and temperate conditions. Finally, different methods for measuring blood volumes and Hb_{mass} were compared to determine appropriate measures to detect alterations in blood volume compartments for scientific purposes.

The results obtained in each study are discussed in detail in the included manuscripts. This section summarizes the key points of every study aim and highlights future directions for research within the corresponding fields.

3.1 Physiological limitations to exercise in the heat

Previous experimental work suggests that hyperthermia might promote the development of centrally mediated fatigue (Nybo and Nielsen, 2001a). One possible underlying mechanism is suggested to be the hyperventilation-induced reduction in $PaCO_2$ and the concomitant decrease in CBF which could possibly lead to cerebral deoxygenation or increased brain temperature (Nybo and Nielsen, 2001a, Nybo and Nielsen, 2001b). To investigate the association of a reduced cerebral perfusion and hyperthermia-induced fatigue during exercise in a hot environment we administrated small amounts of CO_2 to the inspiration with the aim to prevent the reduction in cerebral blood flow and thereby to test this hypothesis directly.

As hypothesised, maintaining $PaCO_2$ during exercise in the heat prevented CBF from becoming reduced. This, however, did not improve exercise performance arguing against a reduced CBF being a major limiting factor for exercise performance conducted in the heat. Reasons therefor include that cerebral deoxygenation was not severe enough to induce cessation of exercise and also brain temperature most likely did not reach critically high levels. Our results were supported by findings of other studies showing no beneficial effect on exercise performance when CBF was normalized by means of CO_2 supplementation in hypoxic conditions (Subudhi et al., 2011, Fan et al., 2012, Siebenmann et al., 2013) and in in elderly individuals (Flück et

al., 2014). Seemingly, a diminished CBF is of only very limited importance also for exercise performance in heat-stressed humans and possibly rather cardiovascular limitations led to premature fatigue in our study. This, however, does not rule out that, indeed, the CNS may limit exercise performance in the heat. To further understand the complexity of centrally mediated fatigue, future studies focusing on determining brain temperature are needed and should also include prolonged submaximal exercise bouts.

3.2 Exercise performance and heat acclimation

With HA exercise performance in a hot environment gradually recovers compared towards cool or temperate conditions (Sawka et al., 1985, Nielsen et al., 1993, Lorenzo et al., 2010). Although possible candidates have been proposed (Senay, 1975, Kirby and Convertino, 1986, Nielsen et al., 1993), the question regarding underlying mechanisms responsible for this improvement remains not completely understood. Controversy also exists regarding the potential benefits of HA not only for exercise performance in hot but also in temperate or cool conditions (Corbett et al., 2014). The aim of this study was to elaborate on these topics by conducting a mechanistic counter-balanced cross-over HA study investigating physiological adaptations and aerobic exercise performance in well-trained subjects.

As expected, after the HA period PV, CBF and sweating responses were improved. However, they did not correlate with the concomitant gain in exercise performance. In the past, especially the hyperthermia induced expansion in PV has been suggested to facilitate exercise performance (Senay, 1975, Senay et al., 1976, Nielsen et al., 1993, Lorenzo et al., 2010). In our study, however, artificial PV expansion by means of albumin infusion did increase $\dot{Q}$ but not exercise performance in heat stressed humans which is in line with the only previous study that applied a similar experimental approach (Watt et al., 2000). We speculate the reason therefor to be the concomitant hypervolemia-induced hemodilution which likely offsets some potential beneficial effect of the enhanced $\dot{Q}$ on exercise performance in the heat. Nonetheless, this remains to be established. A further approach for future studies would be to elaborate on the idea that dynamic changes in PV, i.e. PV retention during exercise is more important than absolute resting PV (Racinais et al., 2012, Racinais et al., 2014).

A further finding of this study was that, in contrast to exercise performance in a hot environment, 10 days of heat training did not facilitate exercise performance in temperate

conditions in well-trained subjects. Lorenzo et al. proposed that the adaptations occurring with HA would also facilitate exercise performance in temperate conditions even in well-trained individuals (Lorenzo et al., 2010). It needs to be mentioned that in the study of Lorenzo et al. subjects in the HA group trained at a relative higher intensity compared to the subjects of the control group which could in part explain the difference of their and our results. Our findings were confirmed by a study conducted by Karlsen et al. who found no alterations in normothermic $\dot{V}O_2max$ or outdoor Time Trial performance after HA in competitive cyclists (Karlsen et al., 2015). Whether HA increases exercise performance in cool and temperate environments is still debated (Nybo and Lundby, 2015), however, the body of evidence supports the conclusion that well-trained athletes benefit from exercise training in the heat if also competing in the heat, but that this is not the case if the competition is held in normothermic conditions or below. For untrained individuals, in contrast, this might be different.

3.3 Determination of blood volume compartments

Various methods exist to measure blood volumes and Hb_{mass} and often different approaches are used for detecting the different blood volume compartments, i.e. the CO re-breathing method for Hb_{mass} and the ICG method for PV. The last aim of my PhD project was to compare the most commonly used approaches to determine blood volume compartments and to test the hypothesis that one blood volume compartment can be indirectly estimated by the determination of another compartment. A further aim was to determine the most feasible and precise method for detecting changes in blood volumes and Hb_{mass}.

In a first step we demonstrated that if the common CO re-breathing is conducted in the seated position, the administrated CO is not evenly distributed in the blood circulation due to blood pooling in the large leg veins, as indicated by a different carboxyhemoglobin (%HbCO) value in the arm and the leg. This most likely applies to all dilution based methods that are conducted in a resting seated position. We could overcome this shortcoming by adding light cycling exercise to the CO re-breathing protocol which facilitated venous return and therefore promoted complete blood mixing. Accordingly, also the resulting Hb_{mass} was higher in the exercise trial as compared to the seated trial. However, since the CO re-breathing procedure, when combined with exercise, is not easy to conduct, we decided to replace exercise by passive tilting in future projects to induce the same mixing effect.

In a second step, methods for measuring different blood volume compartments were compared, namely the CO re-breathing (the common and the short version), the ICG and the SoF method. The short CO re-breathing version was once conducted in the seated position and once with combined tilting. In support of our previous findings (Keiser et al., 2013), in the seated position CO was not evenly distributed throughout the body which, however, could be improved with passive tilting. Surprisingly, Hb_{mass} was lower in the tilted as compared to the seated version and the reasons therefore remain unclear.

When the CO re-breathing was compared to the ICG method PV did not differ between the two methods when a systemic/venous hct correction factor of 0.91 was applied. Further, it is important to mention that the reproducibility of PV was higher in the CO re-breathing than in the ICG method as indicated by the lower typical error (%TE). In contrast, when comparing RBCV derived by the CO re-breathing and the SoF method, the SoF method revealed lower RBCV values as compared to the CO re-breathing. Although the true volumes will remain unknown, the SoF based values seem at the lower end of what can be expected in healthy young men whereas the values from the CO re-breathing seem to be more plausible. Additionally, it needs to be mentioned that %TE of the CO re-breathing method was less than half of the %TE of the SoF method. Therefore, together with the above presented findings we conclude that the CO re-breathing seems to be a valid and especially precise method for detecting blood volumes. Particularly in situations where a high reproducibility is needed for example to track small changes in PV or Hb_{mass} this method can be recommended.

For heat related studies often changes in PV before and after a HA period are of highest interest, however, recently it has been suggested that also Hb_{mass} might increase with HA (Karlsen et al., 2015). The CO re-breathing method seems to be a feasible and accurate candidate for measuring both. To further improve the CO re-breathing method, future studies could directly compare the CO re-breathing combined with exercise and tilting to verify if tilting induces similar effects as exercise. Furthermore, other measures could be considered to be included in the CO re-breathing procedure to promote uniform CO distribution such as exercise prior to the measurement.

3.4 Closing

Although numerous scientific studies have been conducted in relation to hyperthermia in the past century, yet, much remains controversial or even unknown. The studies undertaken as part of this PhD project, however, have addressed some of these. Even though not all of my addressed questions could be completely answered I have been fortunate enough to add several small but important pieces to the puzzle of different heat related topics. Future studies could consider an even more integrative approach, as suggested by Nybo et al. (Nybo et al., 2014) to further explore the extreme complex but interesting field of hyperthermia related topics.

From a personal perspective I would be exited to peruse the further development of the "perfect" CO re-breathing approach. Beside of that, I hope that I can apply all the knowledge acquired during my PhD period in an interesting job and directly implement the theory to different population groups such as athletes or patients.

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5. Curriculum Vitae

Personal Information

Name: KEISER

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Place of Birth: Zug, Switzerland

Education

2013 – 2016 **PhD**, Zurich Center for Integrative Human Physiology (ZIHP), Institute of Physiology, University of Zurich, Zurich, Switzerland, Prof. Dr. Carsten Lundby

PhD Thesis: Hyperthermia-induced limitations and adaptations to exercise in the heat

Feb – Apr 2013 **Internship**, School of Kinesiology, University of British Columbia (UBC), Vancouver, Canada, Prof. Dr. Andrew William Sheel

2011 - 2013 **Master of Science in Human Movement Sciences with focus on exercise physiology**, Swiss Federal Institute of Technology (ETH), Zurich, Switzerland

Nov – Jan 2013 **Internship**, Swiss Federal Institute of Sports (BASPO), Magglingen, Switzerland, section for elite sports, endurance group, Dr. Jon Wehrlin

Jun – Sept 2012 **Master Thesis**, Institute of Physiology, Zurich Center for Integrative Human Physiology (ZIHP), Institute of Physiology, University of Zurich, Zurich, Switzerland, Prof. Dr. Carsten Lundby

Master Thesis: Hemoglobin mass assessed by CO re-breathing is underestimated when performed in the seated position

Jan – May 2012 **Internship**, Institute of Physiology, Zurich Center for Integrative Human Physiology (ZIHP), Institute of Physiology, University of Zurich, Zurich, Switzerland, Prof. Dr. Carsten Lundby

2008 – 2012 **Bachelor in Human Movement Sciences**, Swiss Federal Institute of Technology (ETH), Zurich, Switzerland

2002 – 2008 **Gymnasium with focus on Spanish**, Kantonsschule Zug, Zug, Switzerland

1996 – 2002 **Elementary School**, Primarschule Acher, Unterägeri (ZG), Switzerland

Original Publications

1. Preserved pulmonary vasoreactivity despite constantly elevated pulmonary vascular resistance at 3454m.
   Hilty MP, Mueller A, Flück D, Siebenmann C, Rasmussen P, **Keiser S**, Auinger K, Lundby C, Maggiorini M.
   High Alt Med Biol. In submission.

2. Haematological rather than skeletal muscle adaptations contribute to the increase in peak oxygen uptake induced by moderate endurance training.
   Montero D, Cathomen A, Jacobs RA, Flück D, de Leur J, **Keiser S**, Bonne T, Kirk N, Lundby AK, Lundby C.
   J Physiol. 2015 Oct. Epub 2015 Sep 14.

3. Heat training increases exercise capacity in hot but not in temperate conditions: a mechanistic counter-balanced cross-over study.
   Keiser S, Flück D, Hüppin F, Stravs A, Hilty MP, Lundby C.
   Am J Physiol Heart Circ Physiol. 2015 Sep. Epub 2015 Jul 6.

4. Restoring heat stress associated reduction in middle cerebral artery velocity does not reduce fatigue in the heat.
   Keiser S, Flück D, Stravs A, Hüppin F, Lundby C.
   Scand J Med Sci Sports. 2015 Jun. Epub 2015 May 6.

5. Cerebrovascular reactivity is increased with acclimatization to 3.454 m altitude.
   Flück D, Siebenmann C, **Keiser S**, Cathomen A, Lundby C.
   J Cereb Blood Flow Metab. 2015 Aug. Epub 2015 Mar 25.

6. Hemoglobin mass and intravascular volume kinetics during and after exposure to 3,454 m altitude.
   Siebenmann C, Cathomen A, Hug M, **Keiser S**, Lundby AK, Hilty MP, Goetze JP, Rasmussen P, Lundby C.
   J Appl Physiol (1985). 2015 Nov. Epub 2015 Mar 6.

7. Age, aerobic fitness and cerebral perfusion during exercise: role of carbon dioxide.
   Flück D, Braz ID, **Keiser S**, Hüppin F, Haider T, Hilty M, Fisher JP, Lundby C.
   Am J Physiol Heart Circ Physiol. 2014 Aug 15.

8. Kidney-synthesized erythropoietin is the main source for the hypoxia-induced increase in plasma erythropoietin in adult humans.
   Lundby AK, **Keiser S**, Siebenmann C, Schäffer L, Lundby C.
   Eur J Appl Physiol. 2014 Jun. Epub 2014 Feb 15.

9. Hypovolemia explains the reduced stroke volume at altitude.
   Siebenmann C, Hug M, **Keiser S**, Müller A, van Lieshout J, Rasmussen P, Lundby C.
   Physiol Rep. 2013 Oct. Epub 2013 Oct 2.

10. The carbon monoxide re-breathing method can underestimate Hb_{mass} due to incomplete blood mixing.
    Keiser S, Siebenmann C, Bonne TC, Sorensen H, Robach P, Lundby C.
    Eur J Appl Physiol. 2013 Sep. Epub 2013 Jun 16.

Oral Presentations at Scientific Meetings and Universities

"Exercise in the heat – or the exciting story of my research" – imMed retreat, June 1st – 2nd 2015, Solothurn, Switzerland

„Physiological mechanisms leading to exercise enhancement after heat training" – 49. Atmungs- und Leistungsphysiologische Tagung, January 30th – 31st 2015, University of Zurich, Zurich, Switzerland

"Limitations to exercise in the heat" – Seminarvortrag, Institute of Physiology, May 6th 2014, University of Zurich, Zurich, Switzerland

"Cerebral oxygenation does not limit exercise performance in the heat" – "Training and Competing in the Heat" Conference, March 23rd & 24th 2014, Doha, Qatar.

"The common CO re-breathing underestimates total hemoglobin mass" – Integrative Human Cardiovascular Control course, May 6th – 10th 2013, Copenhagen Graduate School of Health Sciences, University of Copenhagen, Copenhagen, Denmark